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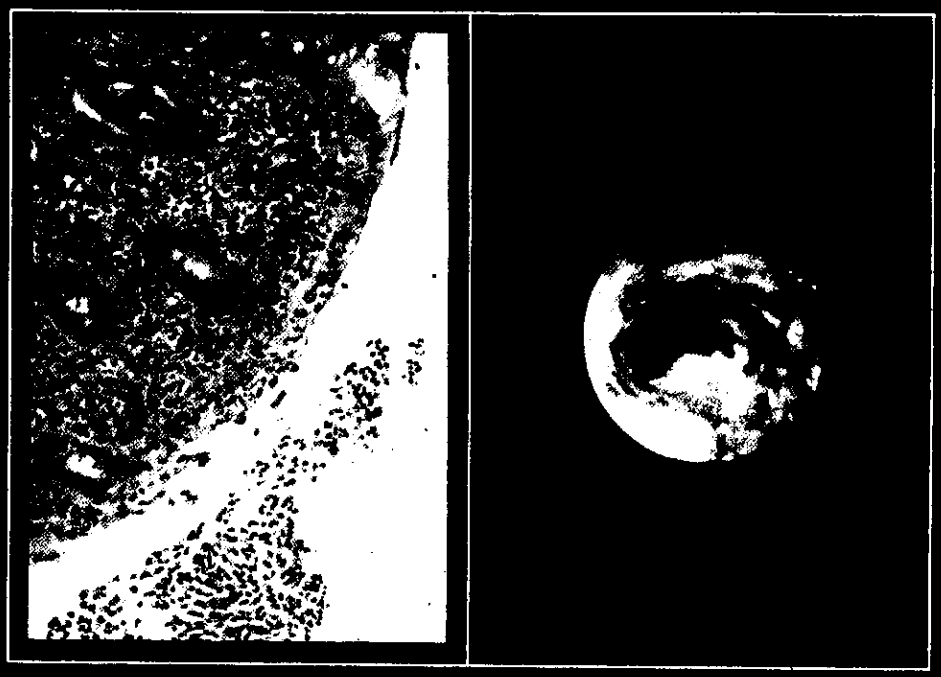

SHIGELLOSIS:

ICDDR,B

SHIGELLOSIS:

A CONTINUING GLOBAL PROBLEM

Proceedings of an International Conference

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Editors:
M. Mujibur Rahaman
William B. Greenough III
Naomi Rock Novak
Shereen Rahman

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PREFACE

The International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) is an autonomous, international, philanthropic and non-profit centre for research, education and training as well as clinical services. The Centre is derived from the Cholera Research Laboratory (CRL). The activities of the institution are to undertake and promote study, research, and dissemination of knowledge in diarrhoeal diseases and directly related subjects of nutrition and fertility,with a view to develop improved methods of health care and for the prevention and control of diarrhoeal diseases and improvement of public health programmes,with special relevance to developing countries. The views expressed in the papers of these proceedings are those of the authors and do not necessarily represent views of the International Centre for Diarrhoeal Disease Research, Bangladesh. They should not be quoted without the permission of the authors and the publisher.

CONTENTS

Introduction

Of all diarrhoeal diseases prevalent in the world, shigellosis is remarkable on several counts. It is an ancient disease afflicting mankind, and has been accurately described since the time of Hippocrates. Due to its propensity to follow famines and break out during wars, shigellosis perhaps was responsible for causing more disability and deaths amongst soldiers in times of battle than did swords or bullets. The disease must have changed the course of history a number of times.

Shigella species is one of the most efficient bacteria known. For as few as 10-100 viable organisms are enough to cause a full-blown clinical attack, compared to more than 100,000 of *Vibrio cholerae* needed to initiate an attack of cholera. As the dominant cause of invasive diarrhoeas, *Shigella* is known to produce severe dehydrating watery motions during the so-called "small intestinal phase", followed by severe colitis or the "colonic phase". Through the inflammatory process in the colon it is responsible for the loss of serum protein directly into the gut. Moreover, it interferes with other colonic functions and suppresses appetite and food intake. Ultimately, shigellosis results in dehydration, electrolyte imbalance, hypoproteinemia and malnutrition. It also causes stunting in children, who may suffer repeated attacks caused by various *Shigella* serotypes, as there is a lack of cross-immunity. *Shigella dysenteriae* type 1 or Shiga bacillus is known to be the most virulent serotype, due to its ability to cause the highest rates of attack and mortality during recorded epidemics and pandemics. In addition, severe complications, such as haemolytic-uraemic syndrome, toxic megacolon etc. have been seen frequently during epidemics.

The incidence of shigellosis and the prevalence of a particular serotype is a fair indication of the state of personal and environmental hygiene prevalent in a community. Since the last World War, improved availability of good quality water and effective isolation of human faeces has been largely responsible, in the developed Western countries, for a dramatic decrease in shigellosis -- followed by the virtual disappearance of its more dangerous serotypes such as *Shigella dysenteriae* type 1 and *Shigella flexneri*. Therefore, there is little urgency in Western countries to pursue research on shigellosis.

The purpose of this International Conference was to kindle an active interest in studying shigellosis, which today is one of the great neglected diseases.

Such study is important, for in the last few years there have been shigellosis outbreaks in many parts of the developing world. It is now clear that for years to come shigellosis will continue to be a major killer of children and old people in poorer parts of the world.

Our attempt to gather together interested researchers to discuss their ideas and to identify areas for *Shigella* research generated an interesting

list of possible topics, that may keep us busy for years to come. This is a modest beginning, and we hope the Proceedings will be a source of ideas to many would be investigators in this old, but still exciting area of scientific endeavour.

M. Mujibur Rahaman

Priority Areas for Research in Shigellosis: Recommendations of an International Conference

EPIDEMIOLOGY AND MICROBIOLOGY

Shigellosis has been and still remains a significant cause of morbidity and mortality in developing countries, particularly in preschool children. From published data and those presented at the Conference it was clear that a sizeable proportion of preschool children, aged between 2-4 years who die due to various aetiological reasons, have a history of dysenteric illness.

In most developed countries of the world, there have been several consistent changes in the epidemiologic characteristics of shigellosis which were also accompanied by a great reduction in its endemicity.

These include:

* Disappearance of both endemic and epidemic strains of *S.dysenteriae* type 1,
* Progressive reduction of *S.flexneri* cases,
* Progressive increase in the percentage of, and often an increase in the number of *S.sonnei* cases,
* Reduction of mortality associated with dysentery.

Though severe illness can result from infection with any *Shigella* strain, the most serious cases of shigellosis result from infection with *S.dysenteriae* type 1, often occurring as epidemics in the developing countries. The nutritional state of the population may have an inverse relationship with outbreaksof shigella epidemics, as demonstrated by its coincidence with famine situations or food shortages. This was documented in many famine conditions.

Aetiologic confirmation of the species and serotypes of *Shigella* is difficult even in well-equipped laboratories; such facilities are rarely available in the developing countries. Therefore, the importance of shigellosis as a cause of morbidity and mortality is often not realised in areas where it is a serious public health problem. For example, a serious epidemic of shigellosis in 1969-1971, caused by a multiresistant strain of *S.dysenteriae* type 1 affecting five countries in Central America, was initially thought to be due to *E.histolytica*, a parasitic illness, almost never seen as the cause of epidemic.

It is recognised that the sensitivity and selectivity of the available medium are not especially high, and some serotypes grow well on one media but poorly on another. As a result *Shigella* is often isolated only if present in very large numbers in samples taken from the environment. There are no satisfactory enrichment or transport media specifically for *Shigella* organisms. Better media, therefore, need to be developed for the isolation and enrichment of *Shigella* species, particularly from contaminated specimens, such as faeces and environmental samples.

Recommendations

* Several hypotheses advanced to explain the changes in the epidemiology of *Shigella* observed in developed countries should be tested in developing countries, so that these changes may result from designed control efforts. In particular, studies should be carried out to assess: (a) duration of excretion, (b) duration of viability in the environment, including food and water, (c) determination of vehicles of transmission, (d) secondary infection rates and disease to infection ratios, (e) reinfection rates and disease to infection ratios. Only when these studies have been completed can the best use of intervention measures, including vaccines, be planned.

* Relationship of *Shigella shiga* epidemics with state of nutrition is an important area of further investigation.

* Better media should be developed for the enrichment, isolation and transportation (storage) of *Shigella* organisms present in faeces and environmental samples. A selective enrichment medium that can be used to detect small numbers of *Shigellae* in contaminated environments is of first priority. Media and isolation procedures that can be readily adapted for use in laboratories and in field conditions should be given priority.

* Surveillance centres and Regional Shigellosis Reference Laboratories, with full-fledged technical infrastructure, should be established in strategic geographic locations throughout the less-developed world, to identify, quantitate and determine the antibiotic sensitivity of *Shigellae* and the global data compared. Field studies should be conducted to develop useful data that can lead to control measures.

* Factors responsible for protecting individuals at risk from *Shigella* infections should be studied in order to clarify the mechanism of "nutritional resistance".

* Dysentery-like symptoms not caused by *Shigellae* require microbiological investigation. The role of *Campylobacter fetus* ssp *jejuni* in causing dysentery-like illness requires further studies.

PATHOPHYSIOLOGY

The major virulence characteristics of the genus *Shigella* are now fairly well-established. To cause disease, the organism must invade the bowel and produce a toxin that is involved in epithelial cell death and, most likely, in fluid loss as well. However, the mechanism of cell invasion is not at all clear, nor is the precise manner in which the toxin triggers fluid secretion.

There are three broad areas of research topics recommended for elucidating the pathophysiology of *Shigellae* infection. Two are basic laboratory studies, namely, (i) the study of invasive properties of *Shigellae* and (ii) characterization and biochemical study of toxin(s) in experimental models. These studies could be undertaken in interested laboratories around the world. The third area of research, i.e. clinical investigation in patients with shigellosis, will require a steady and plentiful source of patients and a sophisticated investigative unit located in an endemic country.

Recommendations

* The mechanism of invasion of the epithelial cells by *Shigellae* is not yet fully understood. There are simple models to study this problem in cell culture which may be effectively investigated now; however, new systems must also be devised for future investigations in the gastrointestinal tract itself.

* The nature of the glycocalyx (glycoprotein coating of the epithelium) and the way in which the organism penetrates it to reach the brush border is yet to be established. This is a difficult problem, and depends on prior work of biochemists on purification and characterization of material from the glycocalyx.

* The role-specific surface or secreted factors responsible for invasion are not known. Are these plasmid controlled? Sophisticated genetic techniques should be applied to characterize organisms in various *in vitro* and *in vivo* models.

* How many toxins do the *Shigellae* produce? Are these the same in non-*Shiga* species? There is some evidence to suggest that one toxin may possess neurotoxic, enterotoxic and cytotoxic properties. Is this due to the same portion of the molecule? Is the mechanism of these effects in different cell systems the same and dependent on the attributes of the cell, or are there different biochemical processes involved?

* Is there a small bowel phase in human shigellosis? Is there colonization, and invasion of jejunum, and production of toxin(s) by *Shigellae?* Clinical investigations to answer these questions, including intubation, perfusion and biopsy techniques, are both feasible and safe. The necessary laboratory methodology to suggest these investigations are available or in developmental stages.

* The possibility of the presence of a toxin-receptor on the jejunal brush border membrane and the colonic cells needsto be investigated. Such studies can be anticipated in the future when *in vitro* and *in vivo* experimental models have been further explored.

* The role of toxin(s) in the pathogenesis of the complications of shigellosis, such as the leukaemoid reaction, haemolytic-uremic syndrome or convulsions, needs to be studied, and their possible presence in the leukocytes, kidney or in the CNS should be investigated. Biological specimens (e.g. CSF) can also be obtained when clinically appropriate and stored for future evaluation. Immunological studies with purified reagents (e.g. monoclonal antibodies) can be planned for future.

* Existing knowledge on nutritional status and effect on dietary intake of chronic shigellosis (symptomatic and asymptomatic) in small children is inadequate.

* Existing knowledge on the relationship of chronic shigellosis to changes in gut flora, absorption, protein-losing enteropathy, nutrient diversion, tissue destruction and catabolism is inadequate, and needs further investigation.

* Protein-losing enteropathy in "asymptomatic carriers" should be investigated further to determine their role in stunting the growth and wastage of nutrients, particularly in children.

CLINICAL COURSE, COMPLICATIONS AND MANAGEMENT

Clinical course of shigellosis may vary from an inapparent carrier state, seen most often with *S.sonnei*, to a fulminant and a common but severe form of dysentery caused by *S.dysenteriae* type 1. The infection may run a mild course, manifested perhaps by some loss of appetite and body weight, to a variety of complications, e.g. severe hypoproteinemia, electrolyte abnormalities (mainly hyponatraemia), convulsions, gram negative septicemia and toxic megacolon. An unusual form of complication has been documented mostly in children aged <7 years suffering from severe shigellosis, caused by *S.dysenteriae* type 1. This is manifested by a high leukocyte count (50,000 - 150,000 polymorphs/mm^3), followed by haemolysis and uraemia, closely mimicking haemolytic-uremic-syndrome (HUS).

Mortality in hospitalized patients with shigellosis has been found to be unreasonably high, varying between 8-15% compared to <0.5% in cholera. Clinical course of *Shigella* infection may be significantly influenced by the nutritional status and specific class of *Shigella* organism.

Recommendations

* The search for new and effective antimicrobials against shigellosis should be continued along with a programme for preventing abuse of antimicrobials.

  -- The role of appropriate antimicrobials in mild and moderately severe shigellosis and on development of the carrier state require further studies.

  -- Factors responsible for early emergence of antimicrobial resistance in shigellosis require identification.

* The effect of experimental antisecretory drugs, such as chlorpromazine, indomethacin, aspirin, etc., on the watery diarrhoea phase of shigellosis deserves evaluation.

* Pathogenesis of convulsion in children with shigellosis should be investigated.

* To reduce the mortality in haemolytic-uremic-syndrome (HUS) caused by shigellosis, the following studies should be carried out:

  -- The role of severe colitis and endotoxemia in the initiation of HUS.

  -- Role of prostacycline in the aetiology and management of HUS.

* Early introduction of food in severe shigellosis with colitis may pose special problems, like development of toxic megacolon, and requires further study.

* The effect of malnutrition on susceptibility to infection and continuation of the carrier state needs investigation. The influence of specific class of shigella organisms on the nutritional status also requires further investigation.

* Impact of shigellosis on the nutritional status in terms of loss of nutrients and stunting should be investigated, in order to assess the impact on growth and inter-relationship with other infectious diseases such as measles.

* The possibility of exclusive breast-feeding conferring immunity to shigellosis should be studied.

* The extent and duration of protein loss in shigellosis of varying severity requires investigation.

* Hospital data (autopsy) combined with those collected through community surveillance ("verbal autopsy") should provide valuable information in determining why and how children die of shigellosis.

IMMUNOLOGY, VACCINE DEVELOPMENT AND INTERVENTION MEASURES

The immunology of shigellosis has not been adequately studied. In light of the recent developments in the field of enteric vaccines (e.g. *Salmonella typhi*) renewed effort should be made to carry out research to develop vaccines against *Shigellae* infection.

While we wait for adequate supply of safe and potable water and improved sanitation and personal hygiene throughout the less-developed world, intervention measures will be needed to cut down morbidity and mortality due to *Shigellae*.

Recommendations

* The streptomycin-dependent *Shigella* vaccines, despite their drawbacks, had shown demonstrable efficacy. Based on these earlier observations, development of new vaccines against shigellosis, utilizing recent technology like the attenuated *Salmonella typhi* vaccine, should be taken up immediately.

* Early safety and immunogenicity tests of the potential candidate vaccine(s) should be carried out in healthy adult volunteers in the developed countries followed by field studies in adults and children in endemic areas.

* Once an effective vaccine is developed immunization of high-risk individuals with oral attenuated *Shigella* vaccines directed against the prevalent serotype(s) should receive priority.

* Little is known on the role of local and cellular immunity in malnourished children with shigellosis and this area requires further attention.

* Little or no knowledge is available on susceptibility to shigellosis of pre-term infants as compared to infants of full-term, small for gestational age and term adequate for gestational age.

* Anthropological studies should be undertaken to specifically identify habits and practices favouring transmission of *Shigella* organisms within and between the families in the developing countries.

* To reduce (interpersonal) transmission of *Shigellae* simple, practical, culturally acceptable intervention measures must be designed to modify personal behaviours and cultural practices. The efficacy, practicability and compatibility of the behavioural modifications should be evaluated within the specific cultural context.

* Identification of simple, practical, culturally-acceptable practices which if dutifully undertaken should result in decreased acquisition and diminished transmission of *Shigellae*, e.g. washing hand with soap or a suitable substitute should be given priority.

* There is need for more knowledge on the mechanisms of transmission in regard to maternal technology and environmental factors, particularly among the rural and urban poor of the developing countries.

* Evaluation is needed to determine the role of improved hygiene technology in the home and treatment (antiseptic) of cases to limit the carrier state.

Chapter 1

The Emergence and the Decline of Epidemics Due to *Shigella Dysenteriae* Type 1 and *S.Flexneri* in Bangladesh Between 1971 and 1978: Some New Lessons Learned

M. Mujibur Rahaman and K.M.S. Aziz

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

ABSTRACT

In the present report, the experience with Shiga bacillus dysentery in Bangladesh was summarized. Rectal swabs and/or faecal specimens from diarrhoeal patients admitted to the hospitals of former Cholera Research Laboratory (CRL, now International Centre for Diarrhoeal Disease Research, Bangladesh, ICDDR,B) since 1962 were cultured for *Vibrio cholerae*, related enteric bacteria and *Salmonellae* and *Shigellae*. Between 1972 and 1976 there were many outbreaks of severe dysentery, some of which were investigated by the authors. In 1970, only 0.6% (26 of 4140) of the indoor patients yielded *Shigella* as a pathogen in the Dhaka hospital; and the rate increased gradually (9% in 1972, 14% in 1973) to reach a peak in 1974, when the rate of isolation of *Shigella* was nearly 20%. The increase was thought to be a consequence of large movement of population during the war of liberation in 1971. A high rate of isolation was maintained thereafter, with considerable seasonal fluctuation demonstrating peaks in early monsoon and post-monsoon periods. *Shigella dysenteriae*, which was rarely isolated before 1971, became more common from 1972. From 1978, the rate of isolation of *S.dysenteriae* began to decline, and later the disease became endemic. Children were the main victims of secondary infections. Infections due to *S.dysenteriae* type 1 were uniformly severe in malnourished as well as apparently healthy adults. The pain due to colitis was severe, and protein-losing enteropathy resulting in hypoproteinaemia was common. Severe dyselectrolytaemia and extremely high levels of polymorphonuclear leukocytes in blood were common complications.

Some of the patients with leukaemoid reaction developed haemolytic anaemia, a drop in platelet counts often accompanied by acute renal failure, and clinical conditions resembling disseminated intravascular coagulation and haemolytic-uraemic syndrome. Mortality was extremely high: the case-fatality at Dhaka hospital was 10% and that in an epidemic in the St. Martin Island was 7%.

Shigella dysenteriae type 1 (Shiga bacillus) was discovered at the end of the nineteenth century,(1) and its outbreak as epidemics had been historically associated with large movements of population, either due to natural or man-made calamities. Despite the fact that Shiga bacillus was frequently isolated in Europe before, during and after the two great Wars, it seemed to have undergone a rapid decline after the Second World War (2). Between 1968 and 1970, a multi-resistant strain of Shiga bacillus caused a severe pandemic in Central America,(3) with importation and spread of cases within the United States of America (3). The pandemic was notable for its ability to cause high attack and mortality rates,particularly in children (4). We summarize here our experience with the Shiga bacillus dysentery in Bangladesh,which seemed to have started following the war of liberation in 1971, as a consequence of large movement of population within the country as well as across the border between Bangladesh and India. Along with the Shiga bacillus, *S.flexneri* also has become a major health hazard in Bangladesh.

MATERIALS AND METHODS

The hospital and treatment centres of the former Cholera Research Laboratory (CRL, now International Centre for Diarrhoeal Disease Research, Bangladesh, ICDDR,B) had been culturing rectal swabs and/or faecal specimens from diarrhoeal patients as a routine procedure since 1962. Although *Vibrio cholerae* and the related enteric bacterias were the main queries of CRL in those days, all swabs were also plated onto Salmonella-Shigella agar and MacConkey's agar in order to isolate *Shigellae* and *Salmonellae* species. *Shigellae* were often isolated from hospitalised patients with diarrhoea, as well as those from community-based studies. Before the war of Bangladesh, however, most of the isolates belonged to the serotype *S.flexneri* (5). After the end of the war in 1971 and return of the refugees from the camps in India, a large increase in the number of cases of severe shigellosis with complications attending the hospital of CRL was noticed (6). Isolation of both *S.dysenteriae* type 1 and *S.flexneri* increased to such an extent that more cases of shigellosis than cholera were attending the treatment facilities with diarrhoea (7).

Between 1972 and 1976, Bangladesh newspapers published many accounts of outbreaks of severe dysentery manifested as "bloody diarrhoea," with a high rate of morbidity and mortality, particularly in children. We investigated some of these outbreaks, the most notable one in 1973, in the St. Martin Island situated on a coral reef in the Bay of Bengal (8).

RESULTS

Beginning from 1974, a series of publications on shigella epidemics and on its clinical features in Bangladesh from the former CRL (now ICDDR,B) came out. For the first time, shigellosis drew the attention of the investigators at the CRL. It was soon apparent that shigellosis is not only breaking out as severe epidemics, but, in contrast to cholera, was a life-threatening illness and caused many clinical complications, some of which had not been previously reported. The relevant and interesting publications are in the list of references. Some aspects of the finding on shigellosis will be presented during the conference. The following is a summary of the various findings of studies carried out at the ICDDR,B on shigellosis.

Epidemiologic features.

In 1970, only 26 (0.6%) out of 4140 indoor patients yielded shigella as a pathogen in the CRL hospital in Dhaka, the rate increased to 9% in 1972 and 14% in 1973, and continued to increase at an extremely high rate, to reach a peak in 1974 when nearly 20% of the patients yielded positive diagnosis of shigella (6). A high rate of isolation of shigella was maintained thereafter, despite considerable fluctuation from one season to another (7). Early monsoon was the traditional high peak-season for shigellosis, followed by a second peak in the post-monsoon and early winter period. In Bangladesh isolation of shigella was always the lowest during the month of February, which coincides with the end of winter. Among all shigella species, *S. dysenteriae* type 1 was rarely isolated in the hospitalised patients before 1971, *S. flexneri* had always been the predominant strain.

The situation began to change in 1972 when *S. dysenteriae* type 1 was isolated in increasing number, and quite often it surpassed the number of *S. flexneri*. The high rate of isolation of *S. dysenteriae* type 1 continued through 1975, 1976 and 1977. In 1978, isolation of *S. dysenteriae* type 1 began to decline and subsequently became an insignificant proportion of total isolates of shigella. Although many patients with *S. dysenteriae* type 1 infection and its associated complications have been seen from time to time since 1978, it is now an endemic disease.

Epidemiologic investigations carried out during the height of the epidemic showed that children were the main victims of secondary infections after the disease was introduced into the family (9). The secondary infection rate due to *S. dysenteriae* type 1 was found to be 29%, and in *S. flexneri* it was 24%. Investigation in the St. Martin island showed an overall attack rate of 33%, highest (53%) being in the age group 1-4 year (8).

Clinical features and complications.

Although shigellosis tends to produce only moderate discomfort in well-nourished healthy adults of western countries, our experience with the disease in Bangladesh was quite the reverse. It is probable that the majority of the infections prevalent in the western countries was due to *S.sonnei*, a relatively less virulent organism. Infections due to *S.dysenteriae* type 1, in our experience, were uniformly severe, not only in malnourished or small children, but even in apparently healthy adults. During the course of the epidemic we had encountered many episodes where well-nourished and hefty young men (policemen, soldiers, farmers) were so severely ill that they were found wandering about the ward, completely naked, delirious (in the absence of high fever) and often in shock. The pain due to colitis was so severe that most children and some adults cried continuously at the time of defaecation. Protein-losing enteropathy resulting in profound hypoproteinaemia was a common feature, and some children were admitted with a total serum protein of less than 3 gm/100 ml. Severe electrolyte disturbances were commonly seen, hyponatraemia (Na<120 mEq/l) being most frequent (10).

Haematological disorders were the most interesting complications found in *S.dysenteriae* type 1 infections. Over 15% of patients with confirmed *S. dysenteriae* type 1 infection showed extremely high level of polymorphonuclear leukocytes in the blood or leukaemoid reaction with counts above 50,000/cmm. Some of the patients had levels exceeding 100,000/cmm, along with immature polymorphs. During the early stage of the epidemic some patients were diagnosed as leukaemic cases and were referred to the general hospitals. Even bone-marrow showed considerable hyperplasia. A small proportion (15%) of patients with leukaemoid reaction also developed haemolytic anaemia and a drop in platelet counts often accompanied by acute renal failure (11). A clinical condition resembling disseminated intravascular coagulation (DIC) and haemolytic-uraemic syndrome (HUS) was found to be a common complication in patients with leukaemoid reaction during the height of epidemic. To our knowledge, the HUS, a disease of unknown aetiology, was for the first time found to be associated with shigellosis. Subsequent studies indicated its relationship with endotoxaemia (12).

Mortality in hospitalised shigellosis was extremely high, despite the availability of effective antibiotics, intravenous fluids, fresh plasma and other supporting measures (13). Our experience in Dhaka hospital showed a case-fatality of 10%, in contrast to 0.5% in cholera. During the St. Martin epidemic, it was noted that in the absence of effective therapy, the overall case fatality was 7%; the highest, 41% in infants and 22% in patients over 50 years of age. In the same epidemic, the maximum fatality (57%) occurred during the second week of illness, in contrast to 21% during the first week (8). General debility, severe hypoproteinaemia, oedema, pneumonia, gram-negative septicaemia, electrolyte depletion, paralytic ileus, toxic megacolon etc., either alone or in combination, were commonly found to be present at the time of death (13).

DISCUSSION

The cycles of epidemics of highly virulent and multiresistant strains of *S. dysenteriae* type 1 accompanied by *S. flexneri* strains in Bangladesh immediately after the chaotic political situation and the War of Independence in 1971 indicated that large movement of population may have triggered the epidemic. The facilities for bacteriological culture of shigella species at the CRL in Dhaka proved to be extremely useful in promoting active research on shigellosis by a number of investigators. The studies carried out on bacteriology, epidemiology, pathogenesis, clinical management, and complications have not only helped to answer many questions on an "old" disease of the humanity but also have raised new questions. Results of investigations carried out by ICDDR,B, made it possible to establish shigellosis as an important endemic disease responsible for high rates of morbidity and mortality. As a matter of fact, subsequent investigations have determined that shigellosis is a much more important cause of mortality and malnutrition than cholera or any other diarrhoea. Since there is no simple therapeutic measure for management of shigellosis such as the oral rehydration available for watery diarrhoea, control of mortality due to shigellosis is going to be much more difficult than mortality due to the other causes of diarrhoea. Improvement of water supply and sanitary facilities, which should reduce the spread of shigellosis will require large capital inputs and will be beyond the capacity of most of the governments of the developing countries for years to come.

The interesting spectrum of clinical symptoms and complications is now the subject of active research at the ICDDR,B and elsewhere. It is hoped that results of these studies will go much beyond solving the immediate problem of shigellosis.

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Chapter 2

Shigellosis in Children in China

Duan Shu-Cheng

Children's Hospital affiliated with Shanghai First Medical College, Shanghai, People's Republic of China

ABSTRACT

The incidence and case fatality rate of bacterial dysentery among children in China has been described. It was found that during the 24-year-service of a hospital, the proportion of patients with bacterial dysentery had been declining with time. The epidemiological, etiological and clinical aspects of shigellosis have been described; and diagnosis, treatment and prevention of the disease have also been indicated. Data on in-patient admission in the hospital showed that 57% of the stool cultures were positive during the first 3 years of liberation of China. Among the positive cultures, 96% were *S.flexneri* and only 4% were *S.sonnei*. The picture during July 1979 to March 1981 showed that 77.5% of the stool cultures were positive for *Shigellae*, of which 70.2% were *S.sonnei* and 29.3% *S.flexneri*. Clinically in children, bacterial dysentery was characterized by sudden onset, accompanied by fever, vomiting, diarrhoea, convulsion, etc. As compared to 1958, the age of patients admitted during 1978-80 with dysentery has decreased, the duration between onset and hospitalization has increased, septic shock accompanying bacterial dysentery has become more common, white counts have showed some differences and complications of the disease have become significant. Diagnosis of shigella dysentery by clinical symptoms, epidemiologic factors, stool culture and SpA methods was discussed. Drugs used for treatment of the disease include: gentamicin, furazolidone, sulfa-drugs together with trimethoprim, berberium, kanamycin, etc. In addition, Chinese herb medicine was found to be quite effective in shigellosis; at times acupuncture was

also reported to work well. Some measures for the prevention of the disease have been suggested.

The incidence and case fatality rate of bacterial dysentery has undergone marked decrease in China after the liberation in 1949 Our hospital was established in 1952. During these 24 years since its establishment, the proportion of patients with bacterial dysentery among the total number of inpatients has been declining, especially during the last 10 years (Figure 1). However, it is still a relatively common disease, and effective means for prevention and treatment should be found.

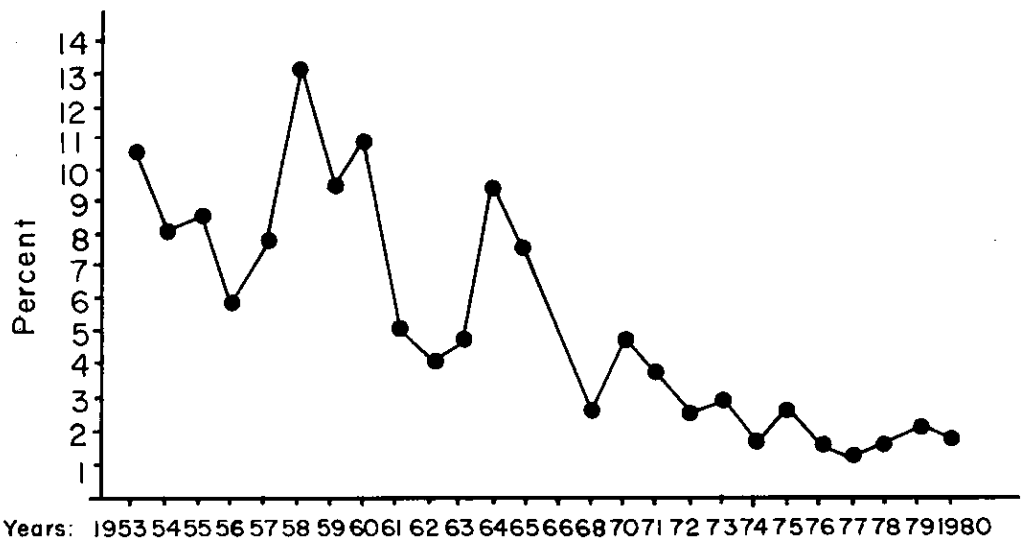

Fig. 1: Percentage of dysentery cases among inpatients during the same period 1953 - 1980.

Epidemiological Aspect.

Statistics in the last 3 years show that in urban districts of Shanghai as well as in our hospital (in both the enteritis clinic and the ward), the great majority of dysentery patients are aged under 4 years, especially during the first and second years of life (Figures 2, 3, Table 1). The disease occurs throughout the year, with peak during the months of July, August and September (Figure 4).

Personal contacts, direct contamination of food or water and flies are the natural ways of transmission of the disease even in institutional outbreaks. In 1980, we treated 2 small outbreaks of bacterial dysentery in kindergartens. The first outbreak occurred in January 5-8, during which there were 22 children with dysentery in the same class, followed by a few more cases after a while. Altogether, there were 28 children in one class,

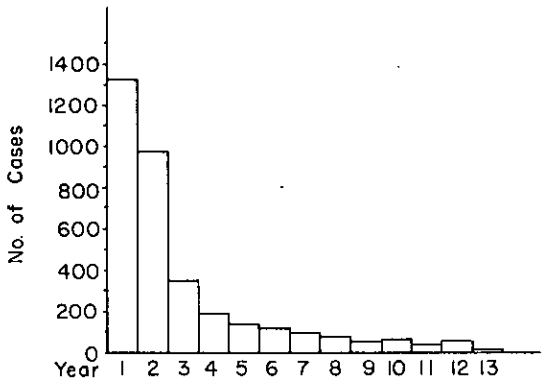

Fig. 2: Age distribution of patients in enteritis clinic 1980

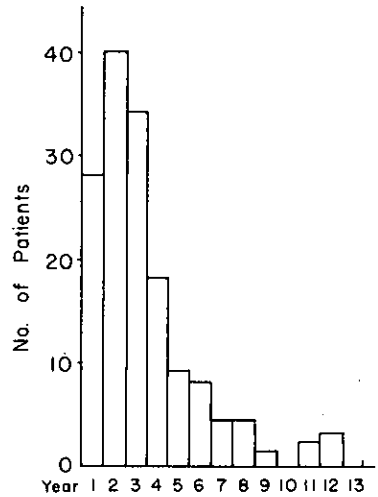

Fig. 3: Age distribution of 151 dysenteric patients with positive stool cultures 1978-80

TABLE 1

AGE DISTRIBUTION OF 307 DYSENTERIC CASES IN WARD 1978-1980

	Cases	0-4 years (%)	0-2 years (%)
1978	84	71 (84.5)	40 (47.6)
1979	120	95 (79.2)	64 (53.3)
1980	103	74 (71.8)	44 (42.7)
Total	307	240 (78.2)	148 (48.2)

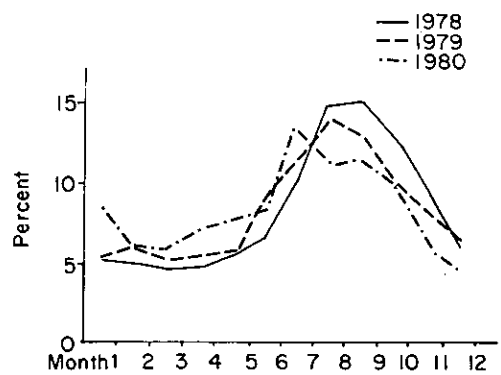

Fig. 4: The distribution of dysentery cases per month in the urban district of Shanghai 1978-80

7 children from another 2 classes and 5 workers affected by the disease (Figure 5). A careful study with phage tests and rectal swab culture of the whole kindergarten and families of some of the patients revealed that the outbreak originated from a boy in the class who had contracted dysentery 2 months ago; his stool cultures were repeatedly positive for F2b. Although he had 3 negative stool cultures before coming back to kindergarten, his rectal swab this time yielded positive result. The pathogenic bacterium was identified bacteriologically, pharmacologically and epidemiologically to be of the F2b type. This epidemic, however, was controlled by appropriate measures.

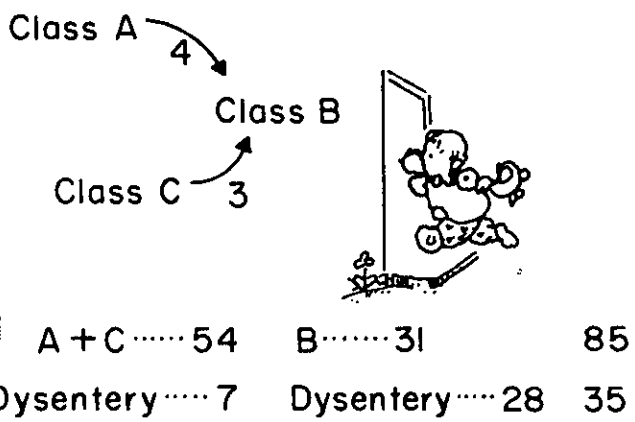

Fig. 5

Chronic bacterial dysentery in children usually recurs during the beginning of the year. Therefore, early diagnosis and effective control of such patients are of great importance in the prevention and treatment of bacterial dysentery.

Another outbreak occurred in May of the same year in another kindergarten. Twenty-three children from the same class became sick with dysentery between May 10 to 19. All positive stool cultures of these 23 cases revealed *S. sonnei*. No other group of children nor any worker was affected by this disease; 3 successive rectal swab tests on all of them showed negative results. Investigation revealed that in this class the 27 children had eaten together from the same batch of food on May 9th, and the container of food for this meal was contaminated by raw vegetables stored in it.

Etiological Aspect.

Data on inpatient admission in our hospital shows that between 1949-1952*, i.e. shortly after liberation, out of 388 stool cultures done, 227 were found to be positive (57%). Among the positive cultures, 96% were *S. flexneri* and only 4% of *S. sonnei*.

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* This data was obtained from the pediatric department of 2 affiliated general hospitals of Shanghai First Medical College, the predecessor of our hospital.

Between 1953-1956 (Table 2), the proportion of cases yielding *S.sonnei* increased gradually.

TABLE 2

MAIN SEROTYPES OF *SHIGELLAE* OF INPATIENTS, 1953-1956

	Cases	Positive cases	Positive rate %	Serotypes				
				Flexneri	*Sonnei*	New casfle	Dispar	Uncertain
1953	344	169	48.0	75	78	1	0	15
1954	310	92	30.0	8	36	2	1	45
1955	377	162	43.0	51	45	0	3	63
1956	?	189	?	78	111	0	0	0

Between 1978-80 (Table 3 and Figure 6) the proportion of *S.sonnei* had increased.

TABLE 3

MAIN SEROTYPES OF *SHIGELLAE* OF INPATIENTS, 1978-1980

	Cases	Positive cases	Positive rate %	Serotypes	
				S.flexneri	*S.sonnei*
1978	84	42	50	24	18
1979	120	54	45	33	21
1980	103	55	53.4	24	31

The shigellosis picture in the outpatients clinic between July 1, 1979 to March 31, 1981 showed that, among a sample 5299 sent for stool cultures for *Shigellae*, 933 cases were positive (17.5%). The distribution of serotypes was as follows:

S.sonnei	725	70.2%
S.flexneri	303	29.3%
S.boydii	5	0.5%

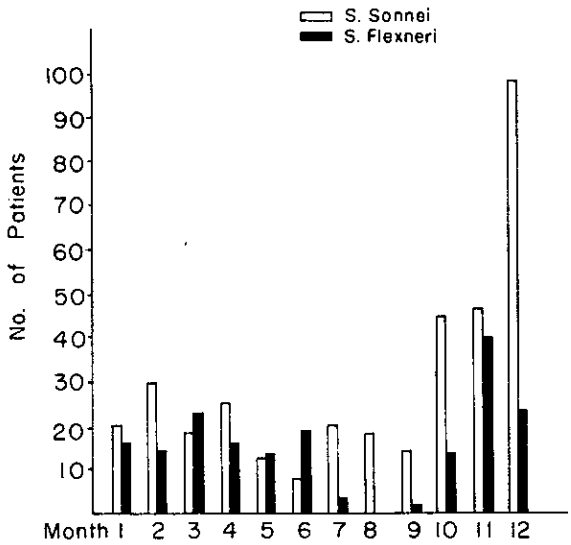

Fig. 6: Number of patients of *S.sonnei* and *S.flexneri* per month (1980)

Data from Shanghai shows that the main serotypes of *Shigellae* found in Shanghai between 1954-1979 are shown in Table 4. Although the data was not complete, yet it is interesting to note the following points.

1. Since 1962 none of *S.Shigae* (*S.dysenteriae* type 1) was isolated.

2. The variations of *S.flexneri* and *S.sonnei:* in 1954, *S.flexneri* occurred in about 83.1%, *S.sonnei* in only 16.1%,

in 1964, *S.flexneri* occurred in about 23.9%, *S.sonnei* 72.8%,

in 1974, *S.flexneri* occurred in about 77.3%, *S.sonnei* in 21.4%.

Since 1975, incidence *S.sonnei* increased gradually.

Clinical Aspects.

In children, bacterial dysentery is characterized by sudden onset, accompanied by fever, vomiting, diarrhoea, convulsion, etc. There is great variation in the severity of the disease and presentation of symptoms. The toxic type of dysentery is becoming less frequent in recent years and the death rate has been greatly reduced. Review of last 10 years mortality showed no death during the last 4 years in our hospital. In the 6-year period before that only 1 or 2 deaths occurred per year.

As compared with incidences in 1958, the toxic type of bacterial dysentery in our hospital has undergone the following changes:

(1) The age of patients admitted with dysentery has decreased. They were mainly between 2-to-5 years of age in 1958, but of the 44 cases of toxic dysentery among 307 dysenteric inpatients in 1978-1980, 31 patients had an age range of 0-4 years, among which 16 patients were under 2 years.

(2) The duration between onset and hospitalization has increased. During 1958, 75 children contracted toxic bacterial dysentery. Among these, 8 children were admitted within 3 hours of onset, 57 children within 12 hours and 10 children within 24 hours. During 1978-1980, there were 44 cases of toxic dysentery. Among these 4 children were admitted within 6 hours, 16

TABLE 4

SEROTYPES OF DYSENTERIC BACILLI OF SHANGHAI (1954-1979)

	No. of specimens	Serotypes				
		S.shigae (%)	*S.schmitz* (%)	*S.flexneri* (%)	*S.boydii* (%)	*S.sonnei* (%)
1954	1196		10 (0.84)	995 (83.1)		191 (16.1)
1955	6800		100 (1.47)	4275 (62.9)		2425 (35.7)
1956	6040	1 (0.02)	74 (1.23)	3461 (57.3)	18 (0.3)	2486 (41.2)
1957	10858	6 (0.1)	73 (0.67)	7853 (69.3)	35 (0.3)	3289 (29.1)
1958	17235	5 (0.03)	125 (0.73)	9938 (57.7)	51 (0.3)	7019 (40.0)
1959	2607	30 (1.15)	34 (1.34)	1522 (58.4)	2 (0.08)	1019 (39.1)
1960	2283		74 (3.25)	1384 (60.6)	60 (2.6)	765 (33.5)
1961	289	2 (0.64)	20 (6.92)	145 (50.2)	15 (5.2)	107 (37.0)
1962	791	3 (0.4)	74 (9.40)	216 (27.3)	12 (1.5)	486 (61.4)
1963	1494		67 (4.49)	488 (32.7)	2 (0.13)	937 (62.7)
1964	2577		85 (3.29)	615 (23.9)	2 (0.1)	1875 (72.8)
1972	342			202 (59.1)	2 (0.6)	138 (40.4)
1973	1421		2 (0.14)	1258 (88.6)		161 (11.3)
1974	3447		32 (0.72)	2442 (77.3)	20 (0.5)	953 (21.4)
1975	3464		15 (0.43)	2176 (62.8)		1272 (36.7)
1976 to 1978	4537		24 (0.52)	3018 (66.5)	12 (0.3)	1483 (32.7)
1979	6443		21 (0.32)	3778 (58.6)	55 (1.1)	2587 (40.2)

within 12 hours, 18 within 24 hours and 6 within 2-4 days. In the last group, the patients had been treated outside of the hospital without improvement before transfering to our hospital on account of the grave condition of the patients.

(3) During 1978-1980, septic shock accompanying bacterial dysentery was more commonly seen. There were 37 cases, 12 of them were of the mixed type. During 1958, neurological symptoms were more prominent. In the 75 cases, 65 had convulsions, 48 coma and 19 semicoma.

(4) Among the inpatients of 1978-1980, 33 cases had a white count of less than 10 thousand, the lowest figure being 3600. Only 11 cases had a white count of more than 10 thousand. This result is rather different from those usually reported.

(5) Complications of the disease: Circulatory shock, acute cerebral odema, sometimes accompanied by respiratory failure, toxic encephalopathy, loss of vision, etc.

Cases of chronic bacterial dysentery have been rare. We had 7 cases in the last 3 years. The disease was not correlated with malnutrition.

Diagnosis.

Clinical symptoms,

Epidemiological factors.

Routine examination of patients' stool: Character, microscopic examination — leucocytes 10-15 per each high power field, usually erythrocytes and macrophages can also be seen, then clinical diagnosis of bacillary dysentery can be established, and finally confirmed by positive stool culture of *Shigellae*.

But even this may not be enough, especially in small infants. For frequent defaecation may also lead to increase of leucocyte count in stool, which does not necessarily indicate bacterial dysentery. Sometimes other kinds of infection may also produce dysenteric-like stool. On the other hand, stool of dysenteric patients may not show typical changes. So it is desirable to improve the reliability of the method of stool culture and to design new methods for early, rapid and more effective diagnosis.

In our hospital, we use SS and McConkey agar plates. We endeavour to improve the quality of stool cultures by concentrating on fresh samples, either collected directly from faeces or by rectal swab. For inpatients with dysentery, two stool cultures should be sent within the first twelve hours after admission, and direct bed-side inoculation is demanded.

For the early etiological diagnosis of shigellosis, we use the SpA (protein A of *Staphylococcus aureus* cowon I which was marked with specific

antibodies) method, 120 stool samples of dysenteric patients had been tested. The concurrent rates of positive culture by means of SS and McConkey agar plates were 71.7% and 77.5%, respectively (Tables 5 and 6).

TABLE 5

COMPARISON OF POSITIVE RATES OF THREE METHODS IN 120 CASES

	McConkey agar	SS agar	GNerrich-ment broth	Total	χ^2	P
S.sonnei (+)	34	33	39	106	0.83	>0.05
S.flexneri (+)	15	21	5	41	10.79	<0.01
Negative	71	66	76	213	1.72	>0.05
Total	120	120	120	360	9.488	<0.05

TABLE 6

COMPARISON OF POSITIVE RATES OF FOUR METHODS IN 120 CASES

	SS agar	McConkey agar	GNerrich-ment broth	0.85% saline	Total
Positive	41	38	36	15	130
Negative	59	62	64	85	270
Total	100	100	100	100	400

Treatment.

We prescribe the most effective drug, preferably per os, and insist on going through the complete course of medication of no less than 6 days. By persuasion, about 90% of the patients in enteritis clinic were able to finish the full course. After stoppage of medication we took other stool cultures. If the result of stool cultures is negative for 3 successive times, we consider that the illness has been cured and notification will be given to parents that children may return to their institutes.

Drugs usually prescribed are gentamicin, furazolidone, sulfa-drugs together with TMP, berberium, kanamycin, etc.

Also Chinese herb medicine is quite effective in shigellosis; at times acupuncture likewise works well.

Drug sensitivity test: By means of microplate method, the sensitivity of 16 drugs for 115 strains of *Shigellae* was done in our hospital in 1980. Of the 115 strains, 79 were *S.sonnei*, 33 *S.flexneri* (F2a: 12, F3a: 10, F1a: 5, F16: 3, F4: 2, F6: 1) and 3 *S.boydii*. The results are shown in Table 7 and Figure 6.

TABLE 7

RESULTS OF DRUG SENSITIVITY TESTS OF 115* STRAINS OF *SHIGELLA* TO 16 KINDS OF DRUGS

Drugs	Efficiency rate %			P
	Total	*S.sonnei*	*S.flexneri*	
Gentamicin	90.4	89.9	90.9	>0.05
Polymyxin	89.5	88.6	93.9	>0.05
Furazolidone	87.0	82.4	91.0	>0.05
Phosphonomycin	86.1	83.5	90.9	>0.05
SMZ_{CO}	84.3	78.4	81.8	>0.05
Cephalothin	82.6	78.5	90.9	>0.05
SD_{CO}	77.4	70.9	81.8	>0.05
Sulfocillin	46.1	40.5	63.7	<0.05
Berberium	44.3	44.3	45.4	>0.05
Kanamycin	42.6	45.6	36.4	>0.05
Ampicillin	34.8	27.9	54.5	<0.01
$BL\text{-}P_{1597}$	33.9	20.3	66.7	<0.01
Doxycyline	31.3	21.5	54.5	<0.01
Streptomycin	26.1	17.8	48.5	<0.01
Tetracycline	22.7	13.9	45.5	<0.01
Chloromphenicolum	22.6	16.5	36.4	<0.05

* 3 *S.boydii* were not included.

Prevention.

1. Make full use of all medical organizations and medical personnel to popularize to the mass the knowledge of infectious diseases, including bacillary dysentery.

2. Stress hygiene of the environment, food and drink and personal hygiene.

3. Health examination, including stool culture especially of workers in the food industry, kindergarten personnel, cooks, etc.

4. For the patients of bacterial dysentery: Isolate the patient and start the treatment as early as possible in the enteritis clinic. Ensure full course treatment (by advice, correspondence, home visit, etc.). Three successive negative stool cultures are needed before the patient is allowed to go back to their institute. Those cases having had positive stool cultures will be asked to follow up stool cultures afterward.

5. For instituted pre-school children: Only those with negative stool cultures for *Shigellae* and *Salmonellae* are permitted to enter. Any child discovered to suffer dysentery will be treated as above, and the children of the involved class are to be closely observed, and sterilization is to be enforced.

6. Control of outbreaks of shigellosis.

Discussions

Dr. Imdadul Huq

We, the microbiologists, have been in a very helpless position, as we have limited facilities for enrichment and isolation vital for any investigation on *Shigella*. Actually you have seen from the report on China yesterday how much difference existed between clinically confirmed *Shigella* cases and bacteriological diagnosis. Every day during the last 18-19 years at the ICDDR,B we face the same questions. Doctors are identifying clinically clear *Shigella* cases, but we cannot isolate *Shigella*. This is due to a limited number of very good available media, the very limited number of enrichment media, which I have described. As you have seen, none of them are very good for the isolation, or enrichment of *Shigella*.

During the last few years, we have seen the emergence of antibiotic resistance. Even though we normally use a media widely-used by many laboratories in this part of the world, we isolate very few *Shigella*, resistant *Shigella*. So, we are left with only non-selective media. But some people say that "I can smell *Shigella*". Well, I can't do that.

We are still faced with a problem: we have many typically red colonies, but we don't get *Shigella*-positive results. Then, we face the same question; visually this appears to be *Shigella*, but we cannot confirm it. On the other hand, is it a *Shigella* with a varying biochemical characteristic?

So, we are in a helpless position vis-a-vis the clinicians or epidemiologists we are trying to support in their daily patient treatment and surveillance work. We have the WHO manual, but it is not a final solution to our problems.

Chapter 3

Shigellosis in Central America

Leonardo Mata

Instituto de Investigaciones en Salud (INISA), Universidad De Costa Rica, Ciudad Universitaria "Rodrigo Facio", Costa Rica

ABSTRACT

Shigella infections are very common in Central America, except in Costa Rica and Panama where prevalence rates are low. Shigellosis is the most serious among all the specific diarrhoeas, due to its invasive character, systemic manifestations, severe nutritional impact, and tendency to become recurrent over prolonged periods.

All species of shigella are found in Central America; village studies reveal the occurrence for several serotypes in any single day. The commonest species are *S.flexneri* and *S.sonnei*. The Shiga bacillus is rare at the moment;but an extensive epidemic covered the whole Isthmus in 1969-71. The epidemic Shiga bacillus bear the multiple-drug resistance plasmid; more recently, some strains have been isolated containing the ampicillin plasmid as well.

The differences in mortality due to diarrhoea among the Central American nations, attest to the importance of environmental factors in determining shigella infections. The reductions or increases in diarrhoea mortality recorded in the last 15 years appear to reflect changes in the socio-economic situation in those nations.

INTRODUCTION

Shigella is a unique bacteria because it is virtually adapted to one host, namely man; primates and dogs are occasional victims of this invading organism. Because of limiting host-parasite relationship, *shigellae* depend on direct or indirect man-to-man contact to perpetuate themselves in nature. Thus, maintenance of infection in general population depends on effective faecal-oral transmission; that is, infection is related to personal hygiene and environmental sanitation.

Shigella has the capacity to invade the mucosal epithelial cells where they multiply, resulting in shedding of damaged cells and infectious bacilli. Its virulence varies considerably, a quality favouring infection with just a few bacilli. This property is very important for the survival of an agent known for its vulnerability in the environment. Although shigella is not completely adapted to the human host (it causes disease, often of severe and even fatal course), it induces many mild and occasionally asymptomatic infections, ensuring their maintenance in the community. It is then expected that shigella and shigellosis are very common in Central America and that their incidence reflects the degree of sanitation and socioeconomic development of the various countries involved.

Diarrhoeal disease in Central America.

Except for surveys and epidemiological studies conducted in Guatemala in the late 50's and in the 60's (1,2), and in Costa Rica and Panama in the 60's (3-5), practically no other comprehensive epidemiologic observations have been made in the area.

These observations revealed that the diarrhoeas are the commonest illnesses in the first year of life, and the second after respiratory infections in the second and third year of life (6). If the common colds and other "minor" respiratory infections are removed, the diarrhoeas stand out as the major source of morbidity in infancy and pre-school age. Since the methods of procedure and bacteriologic technique used in the various studies were different, no attempts are made to compare those data. It is reported, however, that diarrhoea morbidity tends to be greater in Guatemala and Honduras than in Panama and Costa Rica.

Such variability in morbidity, acting in conjunction with occurrence of varying severity of malnutrition, is reflected in the different rates of mortality due to diarrhoeal diseases in the Central American countries (Table 1). Guatemala, El Salvador and Nicaragua had the most serious problem around 1975 (7). It is possible that an improvement is occurring in Nicaragua in recent times. Since a significant proportion of all diarrhoea deaths occursamong infants, diarrhoea has the greatest influence on infant mortality, as was demonstrated for Costa Rica (8).

TABLE 1

MORTALITY ATTRIBUTABLE TO DIARRHOEAL DISEASES
(DEATHS PER 100,000), CENTRAL AMERICA

Country	Year	Age, years	
		1	1-4
Costa Rica	1968	1,778	105
	1977	361 (-8.8)*	25 (-8.4)
El Salvador	1969	1,276	205
	1974	1,276 (+12.5)	182 (-2.2)
Guatemala	1969	1,739	978
	1976	1,400 (-2.7)	511 (-6.7)
Honduras	1969	651	210
	1976	708 (+1.2)	161 (-3.3)
Nicaragua	1968	2,091	198
	1977	1,229 (-4.5)	104 (-5.2)
Panama	1968	482	112
	1974	306 (-6.0)	75 (-5.5)

* In parenthesis, percent change.

Epidemiology of shigellosis.

A general description of the epidemiologic behaviour of shigellosis will be made. *Shiga dysentery* will be discussed later. Prevalence studies emphasizing standard and consistent methods in Guatemala showed shigella carrier rates ranging from 5 to 25 (4,9). Curiously enough, shigella prevalence is lesser in Costa Rica and even less in Panama (Table 2) (5,9,10). There is a seasonality for shigella carriers with low rates at the end and the beginning of each year (the "cold" dry season throughout most of the highlands of Central America), and high prevalence rates from May through September, the wet and humid season (9).

While this applies generally to relatively large communities, or to a region or a nation, a different behaviour can be observed in smaller communities or villages studied prospectively. Thus, the introduction of new serotypes or new virulent strains may lead to outbreaks lasting for several months, until enough persons become infected and immune, to diminish or arrest spread of infection (6).

TABLE 2

PREVALENCE OF *SHIGELLA* CARRIERS IN THE CENTRAL AMERICAN COUNTRIES

Country	Population Studied	Number 4 individuals	*Shigella* prevalence and range
Guatemala	7 rural villages, 1956, 1958	7,223	6% (0.5-9.4)
Panama	30+ rural localities, 1966	10,000+	1% (0-13%)

The "seeding of communities" occurs through introduction of new strains from large urban and market centres by adults and older children, who then disseminate infection in their own families (6). The diversity of serotypes found in point-prevalence studies of rural communities attests to the active dissemination of virulent serotypes and strains at any one time (Table 3). For instance, three of six villages studied in 1956 had, on one particular day, 7 different serotypes. Survey was conducted on about 700 children under 5 years (11).

However, since shigella often becomes chronic especially in malnourished individuals (see below), the possibility that healthy and convalescent carriers maintain infection in the community is plausible. In fact, this may explain the "reappearance" of the Shiga bacillus in Central America, as will be discussed later.

Prevalence of shigella is also influenced by age, since most breast-fed infants are quite resistant to shigella infection (12), and they become shedders particularly after the first birthday. But there is a cohort effect in the manner that the various shigella serotypes invade growing children in a community, as clearly shown in the Cauque study (Figure 1) (6).

It is generally accepted that severity varies according to shigella species (17). But this may not be the case among children with nutrition deficits, except perhaps for *S. flexneri*, which often tended to induce a more severe type of diarrhoea, and for the Shiga bacillus, for its unusually high virulence (14).

TABLE 3

SHIGELLA SEROTYPES FOUND IN POINT-PREVALENCE SURVEYS, MARCH-APRIL 1956

Shigella Serotype	Locality					
	Masagua	La Fragua	Sn. Miguel Petapa	Sn. Bartolome M.A.	Sta. Cruz Balanya	Pueblo Nuevo Vinas
A1			1			
2		2	8		1	1
3						1
B1a					2	
1b						10
2a			2			
2b	1	3				2
3			1	2	3	1
4a	1	1			1	
5	2	1	1			
6			3		20	5
C5		1				
7					2	
D		4	3	4	3	7
Total serotypes	3	6	7	2	7	7

Shiga dysentery.

In 1969, a regional epidemic of Shiga bacillus dysentery got underway, first in the border between Mexico and Guatemala, and then spreading into many regions of Mexico, and into most of Guatemala, Belize, El Salvador, Honduras, and Nicaragua (15,16). Eventually, Costa Rica was hit, (Figure 2) but the wave did not extend into Panama (17).

In total, more than half a million cases of dysentery must have occurred from 1969 to 1971 in Central America, and not less than 20,000 deaths were recorded (15-19). The case fatality rate was as high as 10-15% in some villages, when the correct form of treatment was still unknown.

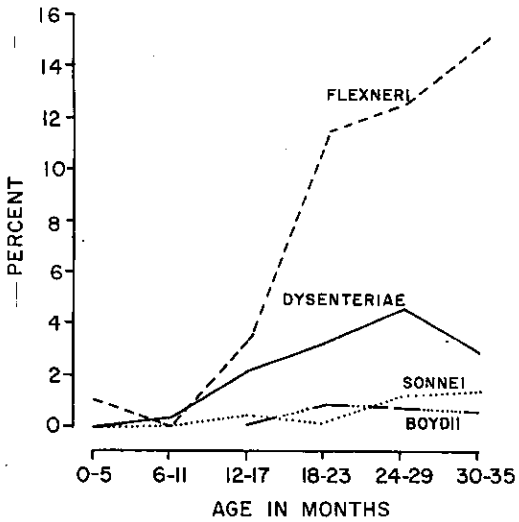

Fig. 1

The dysentery was mistakenly diagnosed as amebiasis with a "complicating virus" (?), because sulfonamide, tetracycline and chloramphenicol, usually successful in treating bacillary dysentery, were fruitless in this outbreak. On the other hand, poor laboratory technique failed to isolate *shigellae* and, concomitantly, inflammatory mucosal cells were misdiagnosed as amoeba trophozoites (15).

Studies in several hundred strains of *S.dysenteriae* type 1 showed that most were resistant to the three antimicrobials above mentioned, plus streptomycin, a characteristic frequently mediated by a transmissible plasmid (Table 4) (15,16,20). Practically all strains of the Shiga bacillus isolated during the epidemic were invasive in the guinea pig's conjunctiva, and possessed several cytotoxins (20). It is hard at this point, however, to fully understand the high virulence and marked pathogenicity of the epidemic strains of *S.dysenteriae* 1.

The Shiga bacillus had been recovered from ill persons and asymptomatic carriers before the epidemic (9,11). Those strains, however, did not possess the multiple-drug resistance plasmid. Like other *shigellae*, the Shiga bacillus elicits a serological response demonstrable by reacting "O" polysaccharide against IgM antibodies from the patients (21,22). The reaction is quite serotype-specific for the Shiga bacillus and other serotypes, and a titre of 1:20 by the passive haemagglutination test is considered significant (21). Extensive testing of sera from a statistical sample of more than 5000 families (in over 180 communities) in Central America, and assuming a titre of at least 1:40 as positive, revealed the presence of *S.dysenteriae* type 1 antibodies throughout the isthmus (Figure 3) (23,24).

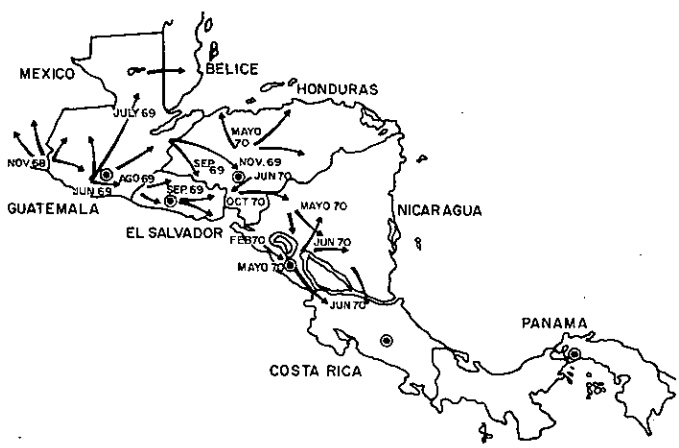

Fig. 2

TABLE 4

CHARACTERISTICS OF STRAINS OF *S.DYSENTERIAE* TYPE 1, 1969-71
CENTRAL AMERICAN EPIDEMIC

Characteristics	Number tested	% with characteristics
Resistance to tetracycline, chloramphenicol, streptomycin and sulfathiazol	180	99.4
Sensitivity to ampicillin nalidixi acid, trimethoprim-sulfa	205	100
Invasiveness of guinea pig's conjunctiva	20	100

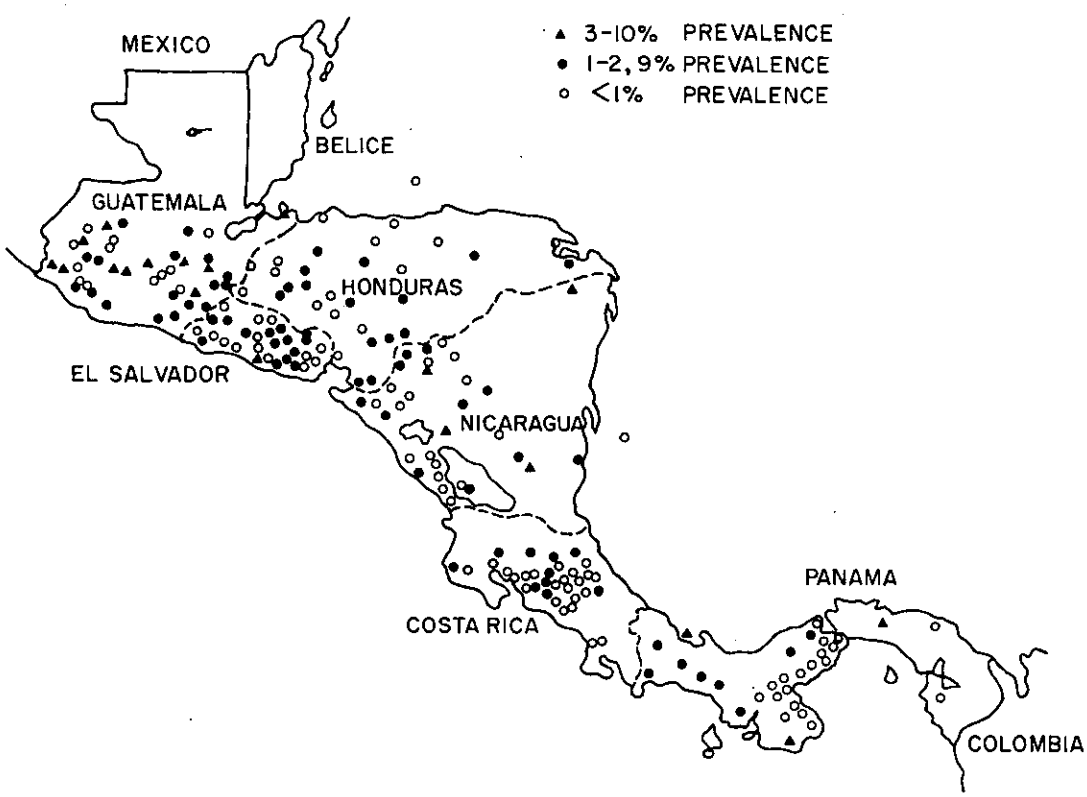

Fig. 3

It is evident that the Shiga bacillus is endemic in the regions and that it is generally not diagnosed, either because it is difficult to isolate in regular media (we prefer tergitol 7 agar with tryphenil-tetrazolium chloride (15)), or because serotyping generally is not done. The reasons for the apparent exacerbation of the organism and for the explosive dissemination in Central America in 1969-71 remain unclear, although several explanations may clarify at least part of the phenomenon (15,16,19).

The epidemic subsided after 1972, although outbreaks in villages and outbursts of cases in hospitals have been noted thereafter; it was abated in Costa Rica where only a few hundred cases and few deaths were reported. Part of the reason for the arrest of the epidemic, especially in Nicaragua and Costa Rica, can be partly attributed to rapid application of the new knowledge of treating severe cases with appropriate antimicrobials. In fact, Costa Rica, a country in transition that enjoys a higher level of hygiene and sanitation than the rest of Central America, benefitted from aggressive campaigns to strengthen mechanisms aiming at diminishing transmission. The epidemic did not progress south of the Central Plateau, and did not reach Panama or South America (17,19,24).

Surveillance systems are in operation in Guatemala and in Costa Rica. In a small outbreak in an Indian Guatuso family, several strains of *S.dysenteriae* 1 resistant to ampicillin were isolated, another complication in the already complex situation of the epidemic Shiga bacillus (25).

Genetic manipulations were made with *S.dysenteriae* 1 strains isolated in this family outbreak (25), in extensive outbreaks in Bangladesh (26), and Mexico (27,28). The results, in Table 5, indicate a distinct homogeneity of the ampicillin plasmid in strains of these organisms recovered in those distant places (24,30). In fact, the nature and biology of the multiple-resistant plasmid also showed to be identical for a variety of strains of different *Enterobactericeae* recovered from different materials, diseases and patients, as this ampicillin plasmid was also found identical to that found in the ampicillin-resistant strains of *Salmonella typhi* of the extensive outbreak in Mexico (31), as well as in many other regions of the body (32). It is evident that the plasmids associated with virulence, being transmitted throughout the world, are the same according to the relatively crude techniques used in molecular biology; but it may be possible that once more sophisticated techniques and molecular epidemiology develop, plasmids of different situations or geographical regions may turn out to be different.

Clinical aspects of shigellosis.

Shigellosis is usually a serious disease, characterized by cramps, multiple bowel movements of watery diarrhoea first, and later multiple small evacuations of mucous and blood and pus; vomiting, prostration, anorexia and fever may also occur, and the severity varies with the species of shigella, with the serotype, and according to the current strain circulating in the community. The Shiga variety is the most serious, as illustrated by the high

TABLE 5

IDENTITY OF Ap^r PLASMIDS OF *ENTEROBACTERIACEAE**

Bacillus carrying Ap^r plasmid	Mol. wt. (daltonx10^6)	N° copies in *E.coli* K-12	Polymerase 1 requirement
S.dysenteriae 1 (Mexico)	5.5	39	+
S.dysenteriae 1 (Costa Rica)	5.5	31	+
S.dysenteriae 1 (Bangladesh)	5.5	30	+
S. panama	5.5	35	+
C. freundii	5.5	35	+

* After Crosa, Olarte, Mata, Luttropp and Penaranda.

attack rate and severity of symptoms recorded at any one time. Leukemoid reactions were described in Central America (33) and in Bangladesh (34). *S.dysenteriae* 1 can be associated with "toxicosis", toxic megacolon, involving the entire organ and even parts of the adjacent small bowel mucosa. The lesion is of coagulation necrosis of the mucosa and sometimes of the submucosa. When there is toxic megacolon, the inflammatory process compromises the *muscularis mocosae*, with varying degrees of destruction of the plexus. Thrombi were found in veins and arteriols of the submucosa and *lamina propria* in all cases of fatal colonic involvement. The common complication was thromboembolism, cortical necrosis and toxic megacolon (35). Intravascular coagulation was found in one quarter of all fatalities autopsied, with lesions in kidneys, adrenals, pancreas and liver.

Nutritional implications.

It was evident from our observations in Guatemala, that shigellosis can easily develop in the well-nourished host, making him very ill and eventually, rendering him weak and postrated, as noted in Guatemalan adults who had naturally recovered from the disease. The pathogenic actions that favour or accentuate malnutrition are obscure, but they are related to cell invasion, tissue destruction, increased fluid and electrolyte secretion, and the presence of enterotoxin in tissues (36,37).

The clinical demonstration that shigellosis has an adverse effect on nutritional status has derived from clinical and field studies. Hospitalized children recovered from malnutrition, easily go into negative nitrogen loss of considerable magnitude during the course of shigellosis (38). More recently, evidence indicates that shigellosis is a protein-losing enteropathy of relative importance (39). In the field, shigellosis is definitely associated with weight loss of long duration, if treatment with oral fluids and required antimicrobial drugs are not instituted (6). Weight deficit and height arrest can persist for weeks and months, and shigellosis can induce prolong wasting (chronic malnutrition), or it can precipitate severe protein-calorie malnutrition, particularly when it occurs coincidental with measles or other diseases (6,38).

Shigellosis, if not treated with drugs, tendsto persist in about one fifth to one quarter of the cases, as chronic recurrent diarrhoea. A study conducted when evidence suggested that antibiotics were not needed, and when effective oral rehydration had not become available, revealed that, all too often, shigella infections become chronic with recurrent symptoms. The significance of chronic shigellosis for the host nutrition has not been elucidated, although knowing the damage inflicted to the mucosa and the accompanying nutrient loss, it is expected to be important.

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Chapter 4

Shigellosis in Poland 1965–1980

H. Stypulkowska -Misiurewicz[1] and J. Kostrzewski[2]

[1]*National Institute of Hygiene,*[2]*Polska Akademia Nauk, Warszawa, Poland*

ABSTRACT

Results of epidemiological investigation of shigellosis in Poland during the years 1965-1980, based on data collected by a routine surveillance system, are presented in the paper. In this analysis special attention has been paid to the changing pattern of the aetiological agent prevalent in Poland. Transition from the dominating *S. flexneri* infections to *S. sonnei* infections was observed during the last 20 years. This was combined with changing frequency of *S. flexneri* serotypes. The proportion of *S. flexneri* type 2a infection has fallen from 80% to about 20%, and the proportion of *S. flexneri* 3a and type 6 for the same period of time increased in the sixties and seventies.

Sporadic foci of rare shigella toxins as infections imported from abroad were limited to primary cases, and no secondary cases have been observed in communities where cases were detected. Among persons infected by rare shigella toxins, 8% were excreting the agent as long as one year after the onset of the disease.

Data collected by public health laboratories from routine bacteriological screening of healthy people in Poland, examined for sanitary purposes, shows that the proportion of inapparent shigella infection increased during the last decade.

The routine surveillance of bacillary dysentery in Poland started in 1919 with obligatory reporting and notification of dysentery incidences and deaths.

In 1957, the National Shigella Centre was established in the State Institute of Hygiene, and the bacteriological methods used for the detection of shigella in public health laboratories have been standardized. Since 1965, data concerning dysentery incidence and death, as well as the results of bacteriological examination of suspected cases, were collected and analysed every year by the public health laboratories of the respective provinces and in the National Shigella Centre, for the country as a whole. In addition, all final reports from dysentery outbreaks investigated by epidemiologists at country and province levels were collected and analysed at the National Shigella Centre.

In 1973, the routine surveillance of shigella infections among healthy food handlers and persons employed in children's institutions was established. More than 1 million persons from all over the country were examined regularly every year. Information concerning number of notified cases in individual provinces and in the country as a whole is published every second week; all other information is published annually.

Results of epidemiological investigation presented in this paper are based on the data collected by the surveillance system presented above.

In Europe, bacillary dysentery are considered synonymous with shigellosis. Shigella infections are endemic in nearly all countries, but incidences may vary depending on various conditions.

In 1970-1975, the annual incidence rates of dysentery in Poland fluctuated from 8.5 to 37.7 per 100,000 population. These figures were a little lower than in some neighbouring countries and a little higher than those reported to WHO by some western and northern countries in Europe (Figure 1).

From 1965 to 1980, no significant change in incidence of dysentery was recorded in Poland, excepting epidemics in 1966, 1971-1972 and 1975 (Figures 2 and 3). The number of symptomatic *S.sonnei* infections increased during epidemic years, which in increasing numbers were isolated from healthy persons in contact with patients. However, isolations of *S.sonnei* from healthy persons screened for sanitary purposes decreased.

The number of *S.flexneri* infections gradually diminished with time, disappearing more quickly among patients than among healthy excretors. The same was seen in *S.boydii* infections, although those never were numerous in this country. The changing pattern of the aetiology of dysentery in Poland observed during the year 1965-1980 cannot be explained by changing reporting system or methods used for laboratory examination (Figure 4).

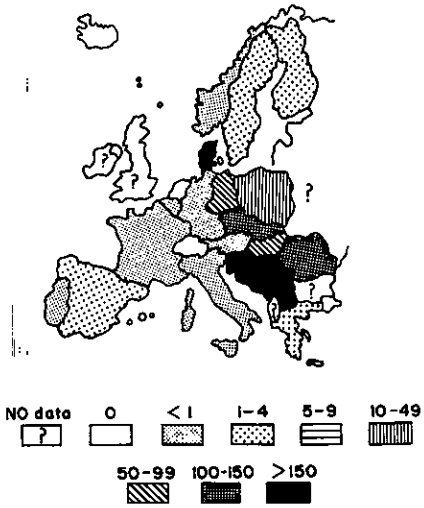

Fig.1: Bacillary dysentery in Europe in 1971 - 1975. Median incidence rate per 100,000 population

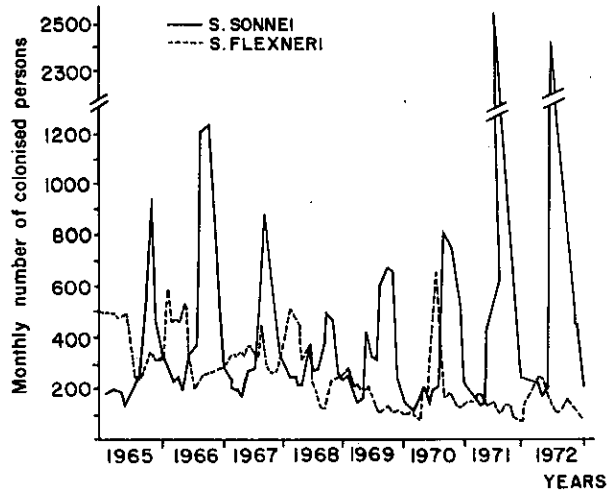

Fig.2: Seasonal distribution of *Shigella sonnei* and *Shigella flexneri* infections

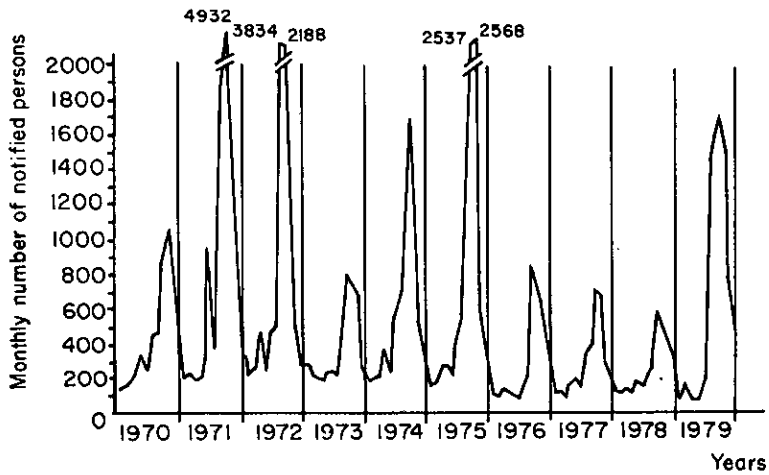

Fig.3: Shigellosis in Poland. Seasonal distribution of cases

Our paper published in 1968 (Arch. Immunol. Ther. Exp. 16, 429, 1968), based on information collected from publications concerning global incidence of dysentery, stipulates a hypothesis that the improvement of sanitary conditions and better nutrition influence the changes in aetiology of dysentery and clinical course of the disease.

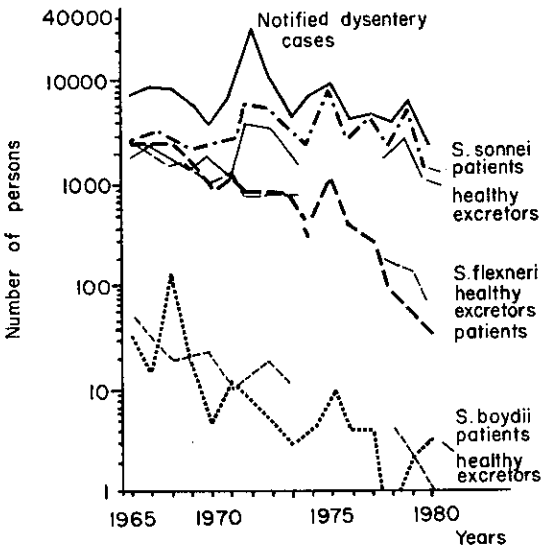

Fig. 4: Changes in the aetiology of dysentery in Poland in 1965 - 1980

Although all three shigella strains (*S.dysenteriae*, *S.flexneri* and *S.sonnei*) were endemic in Europe for decades, however, at the beginning of this century *S.shigae* was a dominating agent attracting the attention of epidemiologists and clinicians.

In 1965, a sharp line divided western European countries, where *S.sonnei* was almost the only aetiological agent of dysentery, and eastern countries, where *S.flexneri* prevailed.

In Poland in 1965, *S.sonnei* prevailed only in 4 regions and 2 big towns. The shift of predominance proceeded from the north and west to the south and east of the country. In 1971, a big epidemic of *S.sonnei* infection spread around the country, and since then *S.sonnei* has prevailed in all provinces (Figure 5).

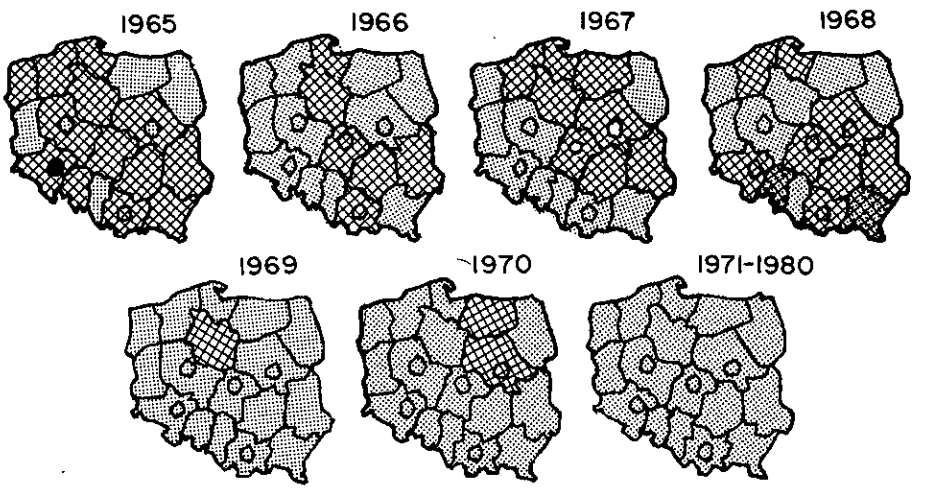

Fig. 5: Regions of *Shigella sonnei* ▦ or *Shigella flexneri* ▩ predominance

S.flexneri infections were prevalent mainly among adult populations, and *S.sonnei* was predominantly seen in children aged up to 14 years (Figure 6). The sanitary measures introduced in 1965-1970 in various institutions for

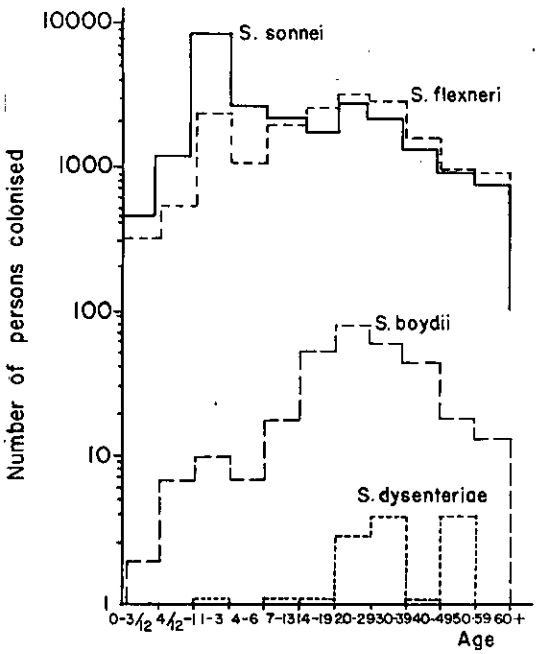

Fig. 6: Distribution of *Shigella* species according to the age of colonized persons

adults, such as psychiatric hospitals and wards or old people's houses, helped to detect chronic carriers, isolate and treat them with anti-bacterial drugs.

Dysentery is reported in Poland throughout the year, but the incidence is much higher in summer and early autumn than in other seasons. There is, however, a distinct difference in the seasonal distribution of *S.sonnei* and *S.flexneri* infections. The highest incidence of *S.sonnei* infections is in late summer and early autumn, and of *S. flexneri* in spring. *S.sonnei* infections, predominant during the last decade, are responsible for the overall seasonal incidence of dysentery in Poland.

Routine serotyping of *S.flexneri* isolated from sick people and healthy excretors demonstrated changes in the frequency of various *S.flexneri* serotypes. Serotype 2a was responsible for 80% of *S.flexneri* infections in 1954. But during the following years the proportion of serotype 2a has fallen to about 20%. The frequency of isolation of serotype 3a and type 6 increased in the sixties and seventies (Figure 7).

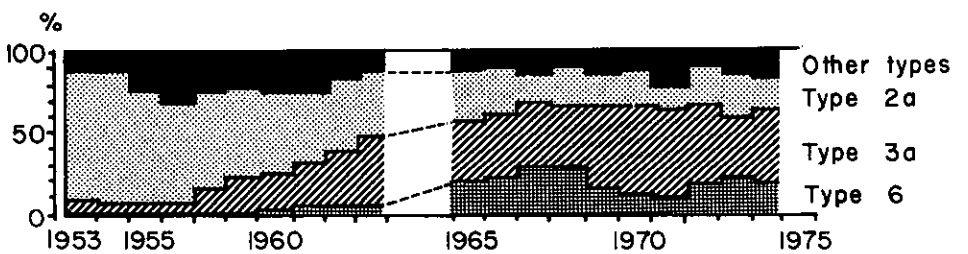

Fig. 7: Changes in *Shigella flexneri* population in Poland in 1954 - 1974

Figures based on data collected from various publications have shown that a similar, though not quite identical, trend was also seen in two other countries, Hungary and Romania (Figures 8 and 9).

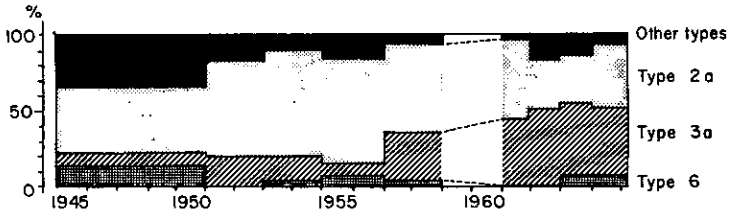

Fig. 8: Changes in *Shigella flexneri* population in Hungary

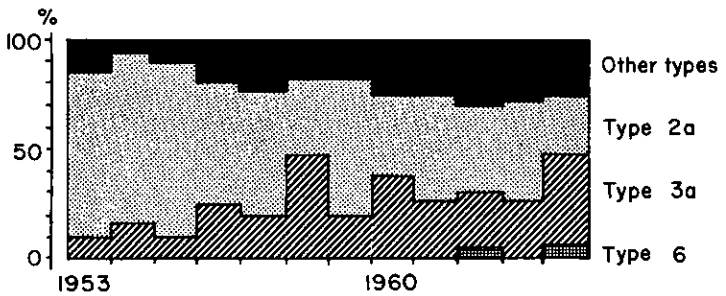

Fig. 9: Changes in *Shigella flexneri* population in Romania

In 1972-1974 seventeen shigella strains were rarely isolated in Poland, some of them only from persons infected abroad (Table 1), e.g. *S. dysenteriae* 1 and *S. flexneri* 5 (Table 2).

In cases of imported infection, no secondary cases were detected,

TABLE 1

ORIGIN OF IMPORTED INFECTIONS BY RARE SHIGELLA TAXONS

Name of the country of origin	Name of the taxon
Egypt	*S. dysenteriae* 1 *S. flexneri* 1b, 5, Y
Libya	*S. boydii* 4
Syria, Turkey, Lebanon	*S. flexneri* 2b
Mongolia	*S. flexneri* 5
Vietnam	*S. dysenteriae* 1 *S. flexneri* X

although all contacts were under strict epidemiological surveillance and subject to bacteriological examination. It may suggest that some shigella strains need special conditions for spreading. To examine the possibility of reinfection in persons infected with rare strains, about 60 percent of

TABLE 2

ANNUAL NUMBER OF PERSONS FOUND TO BE COLONIZED BY RARE SHIGELLA TAXONS

Name of the taxon	Number of colonized persons
S. flexneri 4a	20 - 40
S. flexneri Y	
S. flexneri Y	
S. flexneri 1b	
S. flexneri 2b	2 - 10
S. boydii 1	
S. boydii 4	
S. dysenteriae 2	sporadically
S. boydii 2, 7, 8, 13	found single
S. flexneri 1a, 3b, 3c, 4b, 5	persons

these persons were examined bacteriologically one year after the onset. Only 8 percent continued to excrete the strains. The frequency was higher (14 percent) for symptomless excretors than for former patients (2, 2%).

Periodical excretion of shigella has also been observed.

The frequency of shigella infections among healthy persons examined every year in Public Health Laboratories was nearly ten times higher in some regions than incidences of apparent shigellosis reported through the centralized reporting system (Figure 10).

These findings raise a question: Is our reporting system sensitive enough? And, is the bacteriological screening an efficient tool for detecting shigellosis when anti-microbial drugs are widely used and easily available?

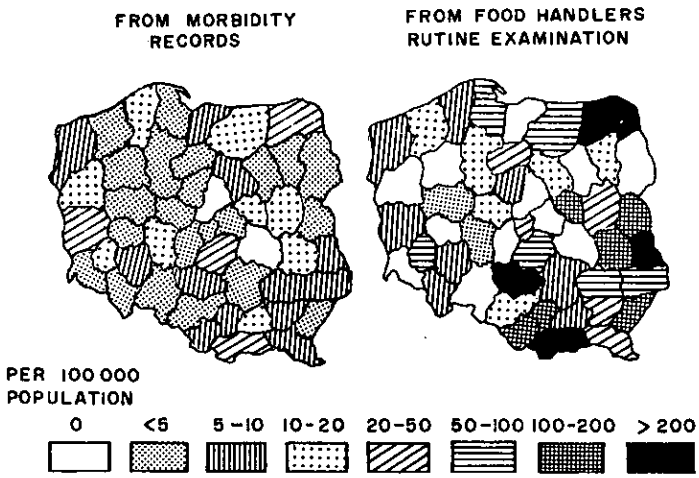

Fig. 10: Shigellosis in Poland:attack rates by regions

Discussions

Dr. Kostrezewski

The surveillance system in Poland started in 1919, with a reporting system of incidence and deaths. So, we had some information for about 60 years in the country. However, for this period of time, there were only very rough figures, as the system was not as well developed as it should have been. In the years after World War II, the rear rank surveillance system improved, and finally, in 1952, the first service system -- Public Health Service -- was built. The Public Health Laboratories, which developed in these few years, were quite efficient; and in 1957 a Shigella Centre was created at the Institute of National Hygiene, Warsaw, which took care of all epidemiological evaluations of the disease.

The rapid increase of shigellosis in the country simply may have reflected the availability of better information. In 1940-50, a predominance of *Shigella flexneri* started, and *Shigella shiga* disappeared. But in the 1950s, especially the late Fifties, *Shigella sonnei* increased in changing proportion, and during the last decade accounted for 95-99% of *Shigella* cases. With this changing clinical pattern the picture became complicated.

The seasonal pattern of *Shigella sonnei* and *Shigella flexneri* showed a seasonal peak and peak of the epidemic and endemic situation, at the end of September for *Shigella sonnei* but not for *Shigella flexneri*. Speaking about flies as carriers of the disease -- flies may pick up *flexneri* and transfer them from stools to human beings, in the Spring for *flexneri* and for *sonnei* during the Summer and earlier. So there seems to be something more than this environmental condition that is responsible for the peak, probably something in the intestine, probably an agent co-relation. What is this? I don't know.

Dr. Mata has mentioned nutritional status as a factor. I would suspect that this factor is of great importance, but it is not yet proved. Moreover, if you look into the phage pattern of *Shigella flexneri* and *Shigella sonnei*, (a difference mentioned in the previous paper) -- that *Shigella flexneri* in this country and in some Central American countries is seen mainly in children -- we find that such is not the case in our country, where *Shigella sonnei* has the highest incidence in the age group 1-4.

The overall incidence of shigellosis in our country is decreasing. *Shigella flexneri* serotypes show a very interesting situation. In the early Fifties we started with a very high proportion of *Shigella flexneri* 2a. During the Fifties and Sixties, the proportion trends and the number of *Shigella flexneri* 2a decreased, and in the early Seventies contributed not more than 20% of all *Shigella flexneri* infections. The *Shigella flexneri* 3a even replaced *Shigella* 2a and *Shigella flexneri* 6. So, which factors are influencing this changing pattern of *Shigella flexneri* serotypes are not clear.

A lot of experimental work done in the past has been on *Shigella* 2a. I was happy to learn from Dr. Formal that this may be done with any other serotypes. I am raising this problem because, though *Shigella flexneri* 2a had a great epidemiological importance in the past, this is no longer so. A situation like that in Poland exists too in other European countries, because the same changing pattern was observed in Hungary, Czechoslovakia, Rumania and elsewhere.

When we look at dysentery as a whole, what are the factors of main concern? What has changed in your countries and in some other countries? When will we observe the change from *Shigella dysenteriae* to *Shigella flexneri* to *Shigella sonnei?* I was fascinated to learn from Dr. Duan how the situation is changing with time in China, because China is located in quite a different environmental and climatic situation compared to Europe. However, the nature of change in Poland is the same, more or less.

Chapter 5

Shigellosis in Japan

Yoshifumi Takeda

Department of Bacteriology and Serology, Research Institute for Microbial Diseases, Osaka University, Japan

ABSTRACT

From the statistics of cases of shigellosis and other enteric diseases reported by the Department of Health and Welfare of Japan since 1900, shigellosis was found to be one of the most common enteric diseases in Japan. Cases of shigellosis increased during 1950's and reached a peak in 1952, and the high incidence was maintained for about 10 years before it started to decrease again. The number of cases reported per year became less than 20,000 in 1968 and less than 10,000 in 1970, and in the following years, the number of reported cases of shigellosis had been more or less constant at 700-1,500. In Japan, there is a special hospital for infectious diseases in each community. The data from the hospitals for infectious diseases in the 11 main cities of Japan showed that shigella was isolated from about 20% of the patients admitted to these hospitals with clinical diagnosis of shigellosis. The distribution of the serotypes of shigella isolated and examined in the major hospitals for infectious diseases showed that strains of various serotypes were isolated, but most of them belonged to serotypes B and D. Investigations showed that there was a tendency for yearly change in the predominant serotype. One of the major reasons for this yearly change was an increase in the number of imported cases of shigellosis. The increase of imported cases of shigellosis was found to coincide with the increase in the number of travellers coming into the country from abroad, mainly from the Philippines, Thailand, Korea, India, Pakistan and Taiwan.

Shigellosis is one of the most common enteric diseases in Japan. Statistics of cases of shigellosis and other enteric diseases have been reported by the Department of Health and Welfare of Japan since 1900, and some of the data are summarized in Table 1. The incidence of shigellosis was more frequent than that of typhoid fever in the early 1900's, but between 1910 and 1920 cases of typhoid fever exceeded those of shigellosis. Although the numbers of cases of typhoid and paratyphoid fever decreased in the 1950's, subsequently cases of shigellosis increased and finally reached a peak in 1952, when almost 12,000 cases were reported. This high incidence was maintained for about 10 years, but started to decrease from 1963. The number of cases reported per year became less than 20,000 in 1968 and less than 10,000 in 1970. During the last 5 years, the number of reported cases of shigellosis has been more or less constant at 700-1,500, which is about twice the number of cases of typhoid fever and about 10 times that of cases of *Vibrio parahaemolyticus* food poisoning.

In Japan, each community must have a special hospital for infectious diseases that accepts only patients with infectious diseases, namely, shigellosis, cholera, typhoid fever, paratyphoid fever, diphtheria, scarlet fever, plague, epidemic cerebrospinal meningitis, typhus, smallpox, Japanese encephalitis, poliomyelitis and Lasha fever. When a case of shigellosis is diagnosed clinically, it is reported to Public Health Service and the patient is sent to the special infectious disease hospital of the community. Table 2 summarizes data from the hospitals for infectious diseases in the main cities of Japan, Sapporo, Sendai, Tokyo, Kawasaki, Yokohama, Nagoya, Kyoto, Osaka, Kobe, Hiroshima and Fukuoka. The number and percentages of isolations of shigella and other enteric bacteria from patients admitted to infectious disease hospitals with the clinical diagnosis of shigellosis has been reported (1). Thus the data in Table 2 give typical figures for this country.

As shown in Table 2, shigella was isolated from about 20% of the patients admitted to these hospitals with a clinical diagnosis of shigellosis. *Salmonella* and *Vibrio parahaemolyticus* were also isolated from many of these patients, and especially in summer months, many patients excreted only *V. parahaemolyticus* (2). This was because infection by *V.parahaemolyticus* usually causes bloody discharges as shigella does, thus resulting in misdiagnosis as shigellosis. Isolation of pathogens from more than half of the patients was unsuccessful. Although these patients were listed as cases of shigellosis, it is possible that some of them did not really have shigella infection. The distribution of the serotypes of shigella isolated and examined in the major hospitals for infectious diseases listed above is shown in Table 3 and Figure 1 (1). Strains of various serotypes were isolated, but most of them belonged to serotypes B and D.

Figure 1, which summarizes the data in Table 3, indicates that there is a tendency for yearly change in the predominant serotype. Between 1967 and 1972, the predominant serotype isolated in these hospitals in Japan was serotype D, *S.sonnei* (Figure 1A). Some strains of serotype B, *S.flexneri* were also isolated and most of them were *S.flexneri* 2b (Figures 1A and 1B).

TABLE 1

NUMBERS OF REPORTED CASES OF VARIOUS ENTERIC DISEASES IN JAPAN DURING THE LAST 80 YEARS

Year	Reported cases of enteric disease				
	Shigellosis	Typhoid fever	Paratyphoid fever	Cholera	*Vibrio parahaemolyticus* food poisoning
1900	46,180	23,846	-	377	-
01	49,384	24,052	-	101	-
02	36,935	21,022	-	12,891	-
03	30,304	18,820	-	172	-
04	22,765	19,628	-	1	-
05	37,981	22,853	-	0	-
06	22,270	25,133	-	0	-
07	24,940	25,916	-	3,632	-
08	32,808	24,492	-	652	-
09	28,005	25,101	-	328	-
10	31,958	35,378	-	2,849	-
11	27,466	34,088	2,112	9	-
12	25,666	31,519	4,046	2,614	-
13	16,777	27,705	3,867	78	-
14	26,121	35,368	6,827	5	-
15	21,136	36,417	7,034	0	-
16	22,449	41,846	6,775	10,371	-
17	14,940	35,176	5,730	894	-
18	13,997	43,072	5,791	0	-
19	12,891	54,595	7,396	407	-
20	12,723	53,756	7,697	4,969	-
21	12,443	49,916	6,286	29	-
22	15,101	52,287	7,108	743	-
23	20,266	52,588	5,288	4	-
24	18,726	58,356	5,330	0	-
25	14,720	45,768	5,052	624	-
26	17,135	43,938	4,451	25	-
30	29,672	41,367	4,467	0	-
35	48,964	37,980	4,173	0	-
40	83,689	40,706	6,251	0	-
45	96,462	57,933	10,059	0	-
46	88,214	44,658	9,154	1,245	-
47	39,219	17,809	4,728	0	-
48	14,665	9,486	2,917	0	-
49	23,961	6,391	2,189	0	-
50	49,790	4,883	1,711	0	-

(to be continued)

(Table 1 - 2)

51	93,039	3,878	1,302	0	-
52	111,709	2,898	835	0	-
53	108,009	2,521	1,098	0	-
54	98,810	2,567	760	0	-
55	80,654	1,939	590	0	-
56	84,437	2,123	509	0	-
57	74,780	2,113	344	0	-
58	81,577	1,901	1,149	0	513
59	85,695	1,546	411	0	737
60	93,971	1,572	319	0	3,994
61	91,538	1,061	213	0	20,536
62	73,999	910	203	0	10,067
63	69,813	995	148	1	12,968
64	52,420	890	148	2	14,263
65	48,621	789	71	0	6,048
66	65,131	893	119	0	7,509
67	30,097	511	139	0	8,806
68	17,792	390	102	0	5,685
69	12,954	417	81	0	11,235
70	9,996	211	50	0	7,922
71	5,833	276	53	0	8,394
72	7,104	304	55	0	10,011
73	3,758	258	48	0	8,021
74	1,719	283	49	0	7,903
75	1,498	524	81	0	15,958
76	727	372	74	0	4,900
77	737	346	77	29	9,629
78	1,037	385	123	34	9,131
79	1,313	391	135	11	11,213

Between 1973 and 1975, there was a significant change, and the rate of isolation of serotype D, *S.sonnei* decreased with increase in serotype B, *S.flexneri* (Figure 1A). Moreover, not only *S.flexneri* 2b, but also other serotypes started to increase. The rate of isolation of *S.flexneri* 3a in particular increased from 1975 to 1977, and that of *S.flexneri* 1b from 1976.

One of the major reasons for this yearly change was an increase in the number of imported cases of shigellosis. Between 1973 and 1975 it was noticed that several patients with shigellosis had just come back from abroad. Thus, the Research Association for Infectious Enteritis started to examine these imported cases of shigellosis. Table 4 summarizes the results of this survey (1). In 1975, only 8.6% of all cases examined at hospitals for infectious diseases were imported cases of shigellosis. However, the rate increased abruptly to 31.8% the following year, and increased further in

TABLE 2

ISOLATION OF SHIGELLA AND OTHER ENTERIC BACTERIA FROM PATIENTS ADMITTED TO INFECTIOUS DISEASE HOSPITALS WITH CLINICAL DIAGNOSIS OF SHIGELLOSIS

		1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980
Number of patients admitted to the hospitals as cases of shigellosis		2372	1506	1086	1634	1215	980	958	867	994	939	708	641
Number of patients from whom pathogens were isolated (% of total isolations)	*Shigella*	594	213	113	505	179	125	85	85	94	173	207	259
		(25.0)	(14.1)	(10.4)	(30.9)	(14.7)	(12.8)	(8.9)	(9.8)	(9.5)	(18.4)	(29.2)	(40.4)
	Salmonella	151	205	122	170	133	88	101	77	159	127	78	60
		(6.4)	(13.6)	(11.2)	(10.4)	(10.9)	(9.0)	(10.5)	(8.9)	(16.0)	(13.5)	(11.0)	(9.4)
	Vibrio parahaemolyticus	147	75	66	106	87	81	79	24	60	73	46	41
		(6.2)	(5.0)	(6.1)	(6.5)	(7.2)	(8.3)	(8.2)	(2.8)	(6.0)	(7.8)	(6.5)	(6.4)
	Enteropathogenic *Escherichia coli*	66	39	66	61	58	36	46	24	34	20	25	22
		(2.8)	(2.6)	(6.1)	(3.7)	(4.8)	(3.7)	(4.8)	(2.8)	(3.4)	(2.1)	(3.5)	(3.4)
	Others	-	-	-	-	-	8	05	49	57	007	46	65
							(0.8)	(1.6)	(56.5)	(5.7)	(12.5)	(6.5)	(10.1)
	None	1412	967	720	795	759	644	638	608	597	441	314	222
		(59.5)	(64.2)	(66.3)	(48.7)	(62.5)	(65.7)	(66.6)	(70.1)	(60.1)	(47.0)	(44.4)	(34.6)

TABLE 3

DISTRIBUTION OF SEROTYPES OF *SHIGELLA* ISOLATED AT INFECTIOUS DISEASE HOSPITALS

Serotype		1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980
A	1					1		1	1	1	1	1			
	2		1	1		1		2	3	1	3	1	2	3	5
	3						1	1						1	3
	6											2			
	7												1		
not determined															1
Subtotal		0	1	1	0	2	1	4	4	2	4	4	3	4	9
B	1a	14	4	18	3	1		1		2	1	7	5	6	8
	1b	29	21	2	2	3	3	2	4	1	5	11	23	26	30
	1c														1
	2							12				2			
	2a	243	548	169	120	43	73	66	73	23	31	19	45	51	66
	2b	31	6	3				12	6	1	1	2	7	8	3
	3a	268	46	27	4	2	4		6	73	8	15	6	11	8
	3b	2	2	3	4		9	3				3		1	2
	3c	6	3	1			1					1		1	
	4	32	9	10	5	1		2	4	4	2	1	4	10	5
	4a												6		2
	4b											1			
	5	1													
	6		2			1				1		1	3	6	4
	x	113	47	3	1	2	2	2		1		1	1	1	
	y	27	27	21	2	4	4	4	3	1	4	1	1	2	1
not determined		2	1										1	1	

(Table 3 - 2)

Subtotal		768	716	257	141	57	96	104	96	107	52	65	102	124	130
C	1	1												1	1
	2	2										1		3	1
	3													3	
	4				1			1			3	3	5		6
	5											2			1
	10														1
	12														2
Subtotal		3	0	0	1	0	0	1	0	0	3	6	5	7	12
D		1805	1328	1106	740	314	829	113	510	123	26	25	55	74	90
Total		2576	2045	1364	882	373	962	222	610	232	85	100	165	209	241

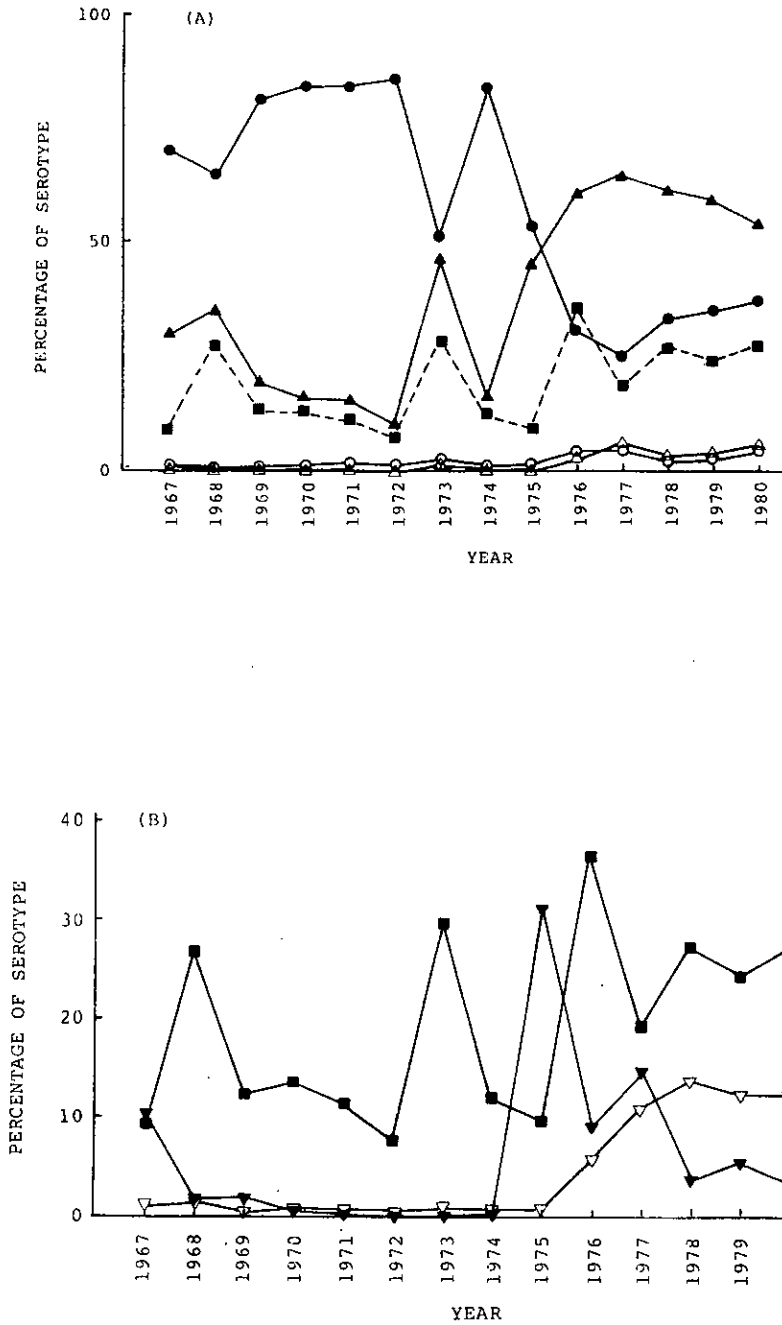

Fig. 1: Yearly change in percentages of isolations of various serotypes.
(A) ○ , serotype A; ▲ , serotype B; △ , serotype C; ● , serotype D;
■ , *S.flexneri* 2a. (B) ▽ , *S.flexneri* 1b; ■ , *S.flexneri* 2a;
▼ , *S.flexneri* 3a.

TABLE 4

SHIGELLA STRAINS ISOLATED FROM OVERSEA TRAVELLERS AT INFECTIOUS DISEASE HOSPITALS

		1975	1976	1977	1978	1979	1980
A	1	1(1)	1(1)	0(1)			
	2	1(1)	2(3)	1(1)	1(2)	3(3)	5(5)
	3						
	6			1(2)			
	7				1(1)		
not determined							1(1)
subtotal		2(2)	3(4)	2(4)	2(3)	4(4)	9(9)
B	1a	0(2)	1(1)	5(7)	4(5)	3(6)	2(8)
	1b	1(1)	4(5)	7(11)	13(23)	9(26)	19(30)
	1c						1(1)
	2			0(2)			
	2a	3(23)	10(31)	7(19)	19(45)	24(51)	41(66)
	2b	0(1)	1(1)	2(2)	4(7)	4(8)	2(3)
	3a	6(73)	3(8)	5(15)	4(6)	7(11)	7(8)
	3b			1(3)		1(1)	2(2)
	3c			1(1)		1(1)	
	4	2(4)	1(2)	1(1)	4(4)	6(10)	4(5)
	4a				4(6)		2(2)
	4b			1(1)			
	6	1(1)		0(1)	3(3)	6(6)	4(4)
	x	0(1)		0(1)	0(1)	1(1)	
	y	0(1)	0(4)	1(1)	1(1)	1(2)	0(1)
not determined					0(1)	1(1)	
subtotal		13(107)	20(52)	31(65)	56(102)	64(124)	84(130)
C	1					1(1)	1(1)
	2			1(1)		3(3)	1(1)
	3					2(3)	
	4		3(3)	2(3)	4(5)		2(6)
	5			1(2)			1(1)
	10						1(1)
	12						2(2)
subtotal		-	3(3)	4(6)	4(5)	6(7)	8(12)
D		5(123)	1(26)	13(25)	35(55)	48(74)	63(90)
Total		20(232)	27(85)	50(100)	97(165)	122(209)	164(241)
% of imported strains		8.6	31.8	50.0	58.8	58.4	68.0

* Numbers in parentheses are those of all isolates.

following years, reaching 68% in 1980. Table 4 shows that several serotypes that had not been isolated for several years in Japan were isolated from these imported cases. These were *S.dysenteriae* 6 and 7, *S.flexneri* 6, and *S.boydii* 1, 2, 3, 5, 10 and 12. The increase of imported cases of shigellosis coincided with increase in the number of travellers coming into this country from abroad (Figure 2).

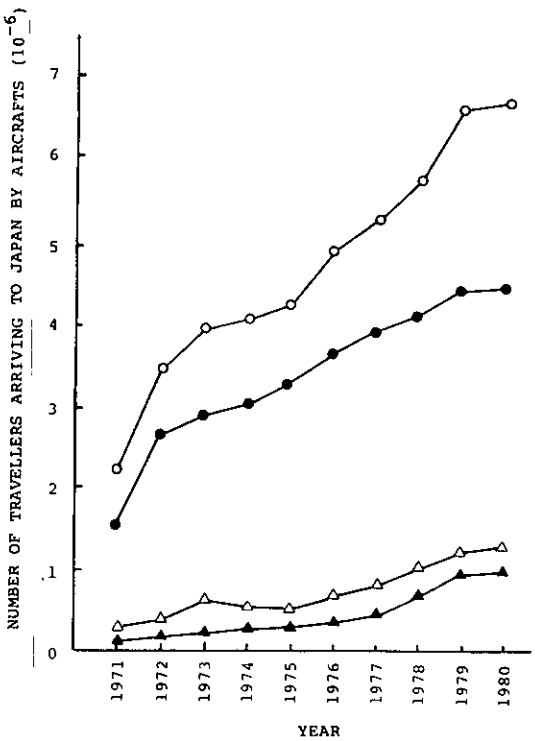

Fig. 2: Yearly change in number of travellers coming into Japan by airplane. ○, Total travellers; ●, number of travellers arriving at Tokyo International Airports (Narita and Haneda); △, Osaka International Airport; ▲, other airports.

The percentages of imported cases of each serotype are shown in Figure 3. Most of the strains of serotype A, *S.dysenteriae*, and

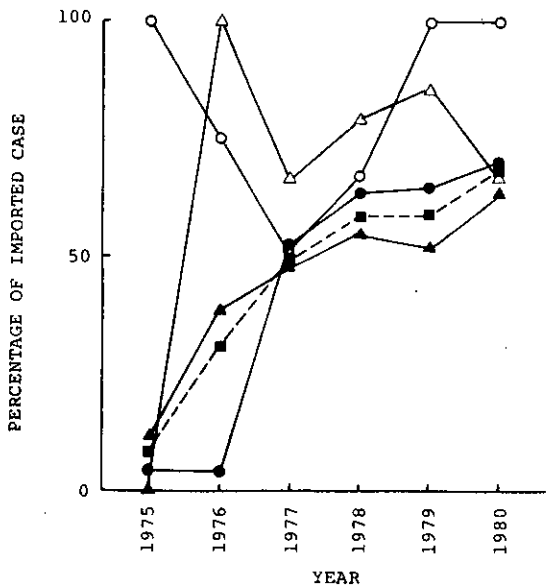

Fig. 3: Yearly changes in percentages of imported cases of various serotypes of *Shigella*. ○, serotype A; ▲, serotype B; △, serotype C; ●, serotype D; ■, total *Shigella*.

serotype C, *S.boydii*, were imported strains, and the percentage of imported cases of serotype B, *S.flexneri*, and serotype D, *S.sonnei* also increased from 1976, reaching almost 60% in 1980.

The survey by the Research Association for Infectious Enteritis (1) showed that the imported strains of shigella were mainly from the Philippines, Thailand, Korea, India, Pakistan and Taiwan.

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Chapter 6

Epidemiological Characteristics of *Shigella* Which Relate to Species and Serotype: Are There Testable Hypotheses ?

Roger A. Feldman[1] and Rickey Wilson[2]

[1]*Chief, Enteric Diseases Branch, Bureau of Epidemiology, Centers for Disease Control (CDC);* [2]*Enteric Diseases Branch, Bacterial Diseases Division, Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA.*

ABSTRACT

Despite decades of study, it was not possible to explain the secular trends observed for shigella. The present article examined the data available for the biological characteristics regarding shigella infection. It was indicated that there is a pressing need to test the hypotheses concerning shigella infection. Studies necessary to test other hypotheses to explain the observations were outlined. Amplifying the data already collected, especially during studies of common source outbreaks, prospective family study, and case control study were suggested to be the three investigative techniques.

INTRODUCTION

Many review articles have commented on secular changes in the frequency of infection with Shigella species and the frequency of infections with the various *S. flexneri* serotypes and subtypes (1-4). These observations have included (1) that epidemics of *Shigella dysenteriae* type 1 have become infrequent, (2) that *Shigella sonnei* are isolated more frequently than *Shigella flexneri* in many developed nations, (3) that *Shigella sonnei* is a prominent cause of diarrhoea in travellers, while remaining relatively uncommon among local residents and (4) that some *Shigella flexneri* serotypes

are isolated more frequently than others. These species and/or serotype-specific differences may relate to differences in the following biological characteristics: (a) infectious dose, (b) disease to infection ratios, (c) duration of excretion, (d) the vehicles of transmission, or (e) survival outside the host. In this article we will examine the data available for these characteristics as they have related to hypotheses advanced to explain these observations and to outline the studies necessary to test other hypotheses to explain these observations.

Secular Trends in *Shigella dysenteriae* type 1 Infection.

Dr. Kiyoshi Shiga, after whom both the genus and serotypes are named, wrote in 1936 (4) that he had no satisfactory explanation either for the observation that epidemics of *S. dysenteriae* type 1 occurred, or for the fact that these epidemics were becoming infrequent in Japan and other countries. He hypothesized that the decrease in number of cases of *S. dysenteriae* type 1 occurred because the carriers were few in contrast to the large number of carriers for both *S. flexneri* and *S. sonnei*. He further hypothesized that the decrease in the number of outbreaks due to *S. dysenteriae* type 1 could be attributed to the inability of this organism to remain as a commensal in the intestine, and suggested that this hypothesis be tested by comparing the duration of excretion of *S. dysenteriae* type 1 to that of *S. flexneri* and *S. sonnei* in an animal model, and also by conducting surveys of the frequency of Shigella carriers in localities where dysentery was present.

The duration of excretion of *S. dysenteriae*, compared to other shigella species, has only rarely been measured. The data of Mata (5) (Figure 1), suggested that *S. dysenteriae* may be excreted longer than *S. boydii* and *S. sonnei*. These data do not explain the observation that *S. dysenteriae* infections are becoming less frequent.

Data concerning case-to-infection ratios tabulated from *S. dysenteriae* outbreaks show that most infections are associated with disease (6) (Table 1). These observations, coupled with the uniformly high age-specific attack rate of *S. dysenteriae* type 1 (Table 2,3,4) (7,8), tend to support the hypothesis that the proportion of *S. dysenteriae* type 1 infections associated with the severe illness is greater than that with other types of shigella, and would suggest that *S. dysenteriae* type 1 is less likely to cause asymptomatic infections. The infectious dose of *S. dysenteriae* (9-11) available from volunteer studies (Table 5) also supports the concept that the carrier state should be rare. Between 100 and 1,000 organisms from an outbreak-related strain, when fed in milk following a bicarbonate feeding, infected half of the volunteers. Unfortunately, there are few data concerning other shigella strains which can be compared to these infectious dose measurements in volunteers. As a result, we do not know how the epidemic character of *S. dysenteriae* relates to a low infectious dose.

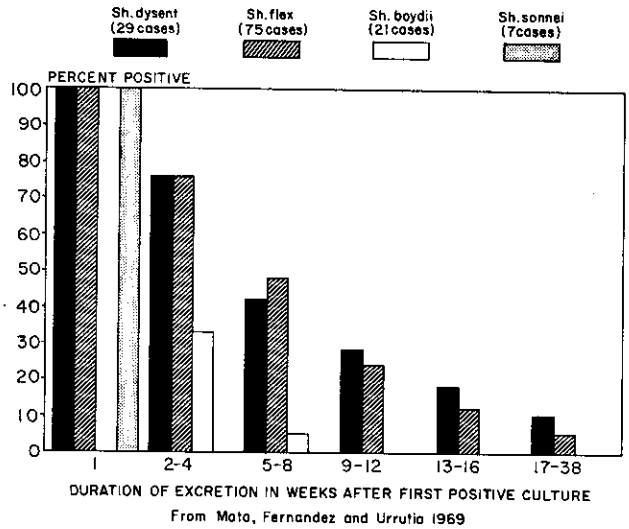

Fig. 1: Duration of Shigella excretion by species, children in Santa Maria Cauque, 1964 - 1967

Other possible hypotheses to explain the differences between *S. dysenteriae* and other shigella serotypes relate to the differences in vehicles of transmission and survival of these organisms outside the host. There are no comparative data to suggest that *S. dysenteriae* type 1 outbreaks are more likely to be associated with water than food, or that the *S. dysenteriae* carrier type 1 is more likely to remain viable in one vehicle than another.

In summary, we have few data to explain the epidemic propensity of *S. dysenteriae* type 1, and none to explain the relative rarity of this strain, with or without epidemics, in recent decades.

Secular Trends of *S. sonnei* and *S. flexneri*.

It has been observed that the frequency of *S. flexneri*-associated illness has decreased, while the frequency of *S. sonnei*-associated illness has either remained constant or may have even increased. Many authors have also observed that *S. sonnei* illness is frequent in travellers to countries where

TABLE 1

CASE-TO-INFECTION RATIO IN 47 FAMILIES,
S. DYSENTERIAE TYPE 1 INFECTION
DACCA, 1974

Age group (years)	Number infected	Number ill	Case-to-infection ratio
0 - 4	15	15	1.0
5 - 9	16	9	0.56
10 +	18	8	0.44

From Khan, Curlin and Huq; Trop. Geog. Med., 1979

TABLE 2

AGE-SPECIFIC MORBIDITY RATE,
S.DYSENTERIAE TYPE 1 OUTBREAK
IN EL COCO, GUATEMALA, 1969

Age group	Number studied	Percent ill
0 - 6 mo	6	0
7 - 23 mo	32	47
2 - 5 yr	97	52
6 - 15 yr	196	31
16 - 45 yr	217	27
46 +	65	31

From Gangarosa *et al;* JID, 1970

TABLE 3

AGE-SPECIFIC MORBIDITY RATE,
S.DYSENTERIAE TYPE 1 OUTBREAK
GUATEMALA, 1969

Age group (years)	Number studied	Percent ill
0 - 4	80	40
5 - 7	65	20
8 - 12	81	16
13 - 19	50	12
20 - 39	72	13
40 +	55	9
All ages	403	19

From Mata *et al;* JID, 1970

TABLE 4

AGE-SPECIFIC MORBIDITY AND MORTALITY RATES,
S. DYSENTERIAE TYPE 1 OUTBREAK,
ST. MARTIN ISLAND, 1973

Age group (years)	Number studied	Percent ill	Case fatality ratio
1	42	41	41
1-4	197	53	9
5-9	212	41	7
5-14	192	34	0
15-49	555	24	0
50+	120	23	22
All ages	1318	33	7

From Rahaman *et al;* JID, 1975

S. flexneri is the commonest cause of shigella infection. Hypotheses to explain these observations have focused on the differences in the vehicles of transmission (12-14). Data relating to differences in vehicles for *S. sonnei* and *S. flexneri* (15,16) infections are difficult to obtain. In the United States, (Table 6) *S. sonnei* and *S. flexneri* were equally common as aetiologic agents for both food-and water-related common source outbreaks. If vehicles are not different, one may hypothesize that the viability of these two species in water or food vehicles may be different. We have no data to test this hypothesis.

Shigella sonnei may be more "host adapted" than *Shigella flexneri*, i.e. asymptomatic infection may be relatively more common, thus increasing the opportunity for spread of the organism. This observation may relate to an increased ability by *S. sonnei* to exist as a commensal in the gut compared to *S. flexneri*. In comparing the frequency of *S. sonnei* infections among shigella isolated in surveys (18-21), with the frequency of *S. sonnei* isolations among shigella isolated from illnesses in diarrhoea studies, it is evident that *S. sonnei* infections are more common than disease surveillance alone would indicate (Table 7, 8). This is in keeping with the suggestion that *S. sonnei* illnesses are less likely to be as severe as *S. flexneri* illnesses. Observations made during a retrospective review of

TABLE 5

RESPONSES OF MAN* TO
S.FLEXNERI 2a AND *S.DYSENTERIAE* 1

Strain	Oral doses**	Number of persons tested	Percent ill
Antibiotic-sensitive endemic strain	200	4	25
A1, *S.dysenteriae* 1	100,000	6	33
Antibiotic-resistant pandemic strain M131	10	10	10
S.dysenteriae 1	200	4	50
	2,000	10	70
	10,000	6	83
Virulent strain 2457T	180	36	22
S.flexneri 2a	5,000	49	57
	10,000	88	59
	100,000	24	58

* From DuPont *et al;* JID 125:12, 1972 and Levine *et al;* JID, 127:261, 1973

** In milk, after bicarbonate

TABLE 6

FOODBORNE AND WATERBORNE COMMON SOURCE
OUTBREAKS DUE TO *SHIGELLA* BY SPECIES
1963 - 1979, UNITED STATES*

Shigella species	Number of outbreaks	
	Foodborne	Waterborne
S.sonnei	33	10
S.flexneri	19	3
S.boydii	1	0

* From CDC Surveillance Data

TABLE 7

COMPARISON OF THE PERCENT DISTRIBUTION OF *SHIGELLA* ISOLATES BY SPECIES FROM GUATEMALAN CHILDREN WITH DIARRHOEA AND FROM SURVEYS OF GUATEMALAN CHILDREN

Shigella species	Percent distribution	
	121* children with diarrhoea	277** children in culture surveys
S. dysenteriae	12	13
S. flexneri	77	62
S. boydii	4	3
S. sonnei	7	22

* From Gordon; Bull WHO, 1964
** From Mata; Rev Biol Trop, 1957
and Beck; AJTM&H, 1957

TABLE 8

COMPARISON OF THE PERCENT OF DISTRIBUTION OF *SHIGELLA* ISOLATES BY SPECIES FROM VIETNAMESE WITH DIARRHOEA AND ASYMPTOMATIC PERSONS

Shigella species	Percent distribution	
	606 persons with diarrhoea	18 asymptomatic persons
S. dysenteriae	7	28
S. flexneri	67	33
S. boydii	9	22
S. sonnei	17	17

From Gaines; Military Med, 1968

shigella surveillance data from Wisconsin (22) supports the hypothesis that *S. sonnei* may be more infectious than *S. flexneri*. Significantly more secondary cases of diarrhoeal illnesses occurred in adults from exposure to index patients one-to-nine years old with *S. sonnei* infections than from exposure to children of the same age infected with *S. flexneri*. This hypothesis might also explain why *S. sonnei* occurred more frequently in travellers to countries where *S. flexneri* is the commonest cause of shigella infections. In two sets of studies, (15,16,20,24), we have data showing a higher percentage of *Shigella sonnei* infections in travellers than in children in the same environment (Table 9, 10). Data from Watt (25) showed *Shigella flexneri* to be more

TABLE 9

COMPARISON OF THE PERCENT OF DISTRIBUTION OF *SHIGELLA* ISOLATES BY SPECIES FROM AMERICAN ADULTS* AND EGYPTIAN** CHILDREN WITH DIARRHOEA

Shigella species	Percent Distribution	
	22** Egyptian children	79* American adults
S. dysenteriae	18	6
S. flexneri	68	62
S. boydii	14	9
S. sonnei	0	23

* From Floyd; AJTM&H, 1956
** From Mohieldin; J.Trop.Ped. & African H, 1965

persistant than *S. sonnei* in the stool (Figure 2); these data do not support the hypothesis that *S. sonnei* is more infectious than *S. flexneri*.

In summary, we have no consistent explanations for the slow, steady decrease in the frequency of *S. flexneri* infection in the developed countries, or for the persistance of *S. sonnei* as the common cause of diarrhoea in those countries where *S. flexneri* is common.

TABLE 10

COMPARISON OF THE PERCENT DISTRIBUTION
OF *SHIGELLA* ISOLATES BY SPECIES
TURKISH* CHILDREN AND
AMERICAN** CHILDREN AND ADULTS
WITH DIARRHOEA

Shigella species	Percent Distribution	
	322* Turkish children with diarrhoea	706** American children and adults with diarrhoea
S. dysenteriae	3	3
S. flexneri	81	29
S. boydii	1	3
S. sonnei	15	65

* From Akman, Turkish J. Ped, 1965,
and **Bergman, Mikro Bult, 1976

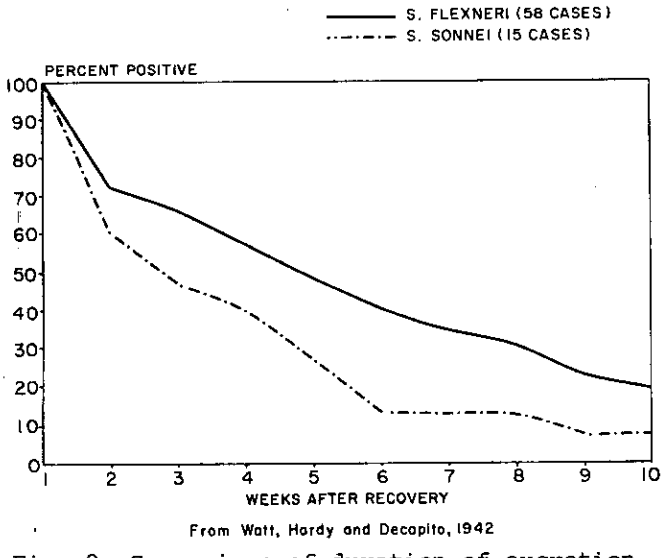

Fig. 2: Comparison of duration of excretion of *Shigella flexneri* and *Shigella sonnei*

Incidences of Infection with Serotypes of *S. flexneri*.

The third set of observations relates to the frequency with which certain types *S. flexneri* are isolated from persons with diarrhoea. The simplified antigen scheme for *S. flexneri* (26) showed six serotypes and additional sub-serotypes (Table 11). It has been reported that certain serotypes of *S. flexneri*, usually 2 and 3 (Table 12), are generally more common than serotypes 1, 5, and 6, (1-3,15,18-20, 23,27-38). In some countries (1) it appears that there are more frequent strains within sero groups (Table 13). There are no data to explain these variations.

TABLE 11

SIMPLIFIED ANTIGENIC SCHEME
FOR *S.FLEXNERI*

S.flexneri serotype	Sub-serotype	Abbreviated antigenic formula
1	1a	I:4 ...
	1b	I:6 ...
2	2a	II:3,4 ...
	2b	II:7,8 ...
3	3a	III:6,7,8 ...
	3b	III:3,4,6 ...
	3c	III:6 ...
4	4a	IV:3,4 ...
	4b	IV:6 ...
5	5	V:7,8 ...
6		VI:-...
X variant		--:7,8 ...
Y variant		--:3,4 ...

(Modified from Ewing and Carpenter 1966)

From Ewing and Carpenter; Int J Syst Bact 1966;16:145-149

DISCUSSION

It is somewhat surprising that we have few satisfactory explanations for the observations reviewed in this presentation. Opportunities, however, exist to amplify the data already collected, especially during studies of common source outbreaks. Under those circumstances, it is possible to study duration of excretion, disease-to-infection ratio, infection rates within families; and subsequently to compare data of the various serotypes and species of shigella.

TABLE 12

PERCENTAGE DISTRIBUTION OF *SHIGELLA FLEXNERI* FROM DIARRHOEAL STOOLS BY SEROTYPE AND COUNTRY

Country	Years studied	Number studied	Percent of each *S. flexneri* serotype					
			1	2	3	4	5	6
China	1954-1960	2473	10	46	26	6	4	4
Guatemala	1955-1961	203	13	15	31	5	4	32
Hungary	1968-1971	4606	8	22	40	16	0	9
Iran	1961-1968	205	6	55	25	2	3	8
Korea	1964	3392	10	23	11	30	25	0
Mexico	1953-1960	186	8	56	7	15	2	8
Nigeria	1960	144	7	65	11	7	5	3
Somalia	1965-1969	228	21	32	11	17	0	18
Turkey	1957-1964	332	15	58	18	4	0	5
U.K.	1972-1978	1284	18	31	17	16	1	13
U.S.A.	1957-1963	198	8	41	20	15	4	12
Vietnam	1968	407	12	53	20	11	1	1

TABLE 13

NUMBER* OF REPORTED ISOLATES AND PERCENTAGE** DISTRIBUTION OF SEROTYPES OF *S. FLEXNERI*, JAPAN, 1951-1965

	S. flexneri Serotypes and Sub-serotypes								
Year	1a	1b	2a	2b	3a	3b	4	5	6
1951	1(1)	30(14)	117(53)	33(15)	11(5)	0(0)	3(1)	1(0)	1(0)
1956	2(1)	10(5)	84(40)	39(19)	35(17)	2(1)	3(1)	0(0)	2(1)
1961	3(1)	14(5)	96(37)	19(7)	74(28)	2(1)	5(2)	0(0)	2(1)
1965	1(1)	3(4)	16(24)	3(5)	24(38)	1(1)	3(4)	0(0)	0(0)

* Number in hundreds

** Percentage in parentheses

From AOKI; Tropical Medicine, 1969

Another avenue of investigation is the prospective family study. A carefully designed family study allows one to make observations similar to those made from common source outbreaks (diseases-to-infection ratio, etc.), and may be more easily performed when common source outbreaks are difficult to identify. The family studies offer the advantage of being able to identify in a natural setting the differences in frequency of occurrence of the various shigella species and serotypes.

A third investigative technique is the case control study. In a well-designed case control study, it should be possible, utilizing either out-patients or inpatients with proven shigella infection, to identify risk factors which might explain differences between the relative frequency of occurrence among the various shigella species.

In summary, despite decades of study, we are unable to explain the secular trends observed for shigella. There is a pressing need to test the hypotheses presented and to develop new testable hypotheses concerning shigella infections.

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Chapter 7

Shigellosis in Dhaka and its Epidemiological Pattern in Affected Families: 1980

Moslemuddin Khan and Md. Shahidullah

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

ABSTRACT

All shigellosis cases attending the ICDDR,B hospital facilities during 1980, and 100 families with one index case of shigellosis were studied to determine the epidemiological and behavioural pattern of shigellosis in Dhaka. Antibiotic sensitivity tests were done to assess resistance patterns. It was found that *shigellae* cases were present throughout the year with higher incidences during April-June. *S.flexneri* was higher in October-January and accounted for 67.5% of the total cases. Though *Shigella* cases were reported from all over the city area, more were seen in the socio-economically poorer areas. Highest attack rates were among the 1-4 year age group, but, contrary to earlier belief, it was also frequent among the less than one-year-olds. Among the family contacts of the index cases, the secondary infection rate was 31.8% and secondary case rate 12.4%; these rates were higher, if *S.flexneri* was the index case. When the index case was below five years, the secondary infection rate was higher. Incidences of *shigellae* were also influenced by the sources of water, types of latrines used, the income of the family and size of dwelling in respect to family size. Seven to 13% of Tetracycline, 2%-3% of Streptomycin, 100% of Sulphamethoxazole Trimethoprim and 100% of Gentamicin were sensitive to *shigellae* during this study.

INTRODUCTION

In 1970, less than 1% of all hospitalized diarrhoea cases at the Centre were due to *shigellae*. This increased after the war of 1971 to 14% by 1974 (1). A high secondary infection and case rate in affected families (2,3) was seen. Resistance to antibiotics and death rates increased. The epidemiology of shigellosis treated in hospital and in contacts of index cases was studied to define this escalating problem.

METHODOLOGY

Hospital and microbiology records of 1980 were analyzed and one hundred families with an index case of shigellosis were followed prospectively. Diarrhoeal history, rectal swabs and left-hand washing (4) were collected daily for 10 consecutive days, and cultured using Salmonella Shigella (SS) and MacConkey plates (5). Contacts having at least one positive culture or three negative cultures were included in the analysis. Contacts with a positive yield of the same serotype as the index were termed "secondary infection", and infected contacts who developed diarrhoeal symptoms were "secondary cases."

RESULTS

Figure 1 shows seasonal variation of diarrhoea and shigellosis cases for 1980. *S.flexneri* occurred throughout the year, peaking from October to January and in May. Other *shigellae* had peaked from April through June. More than 9% of all diarrhoea was caused by *shigellae*. We estimate that more than 11,000 shigella cases attended hospital in 1980. Non-shigella diarrhoea peaked from March to June.

Figure 2 shows that all zones of Dhaka were affected with shigellosis. Except for a high rate in Gulshan P.S., the attack rates were consistent with the previous data on the scatter of other diarrhoeal diseases (6). Some zones had higher rates than others. Over 67% of all shigella were *S.flexneri*, 18% *S.dysenteriae* and 15% *S.boydii* and *S.sonnei* together.

Figure 3 shows incidence of shigella by age, sex and types. It shows that 82% of hospitalized shigella cases were aged less than 5. Thirty-seven percent of all cases were infants less than 1. Although *S.flexneri* predominated, other *shigellae* also was highest in children less than 5. Of the hospitalized cases, the ratio of male-to-female was 57/43. The overall male population of the city was higher than the female population.

From the family study result, the figure 4 shows that the secondary infection and case rates were 43.3% and 27.5% in contacts aged 1-4 years.

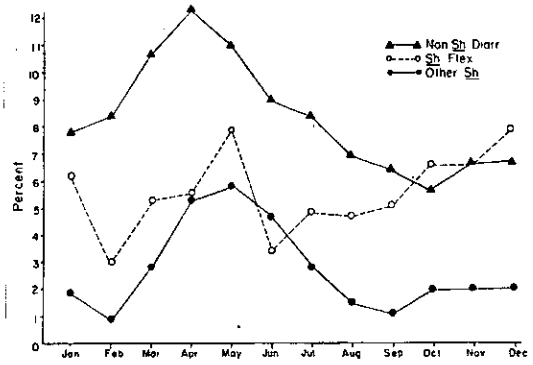

Fig. 1: Seasonal variation of hospitalized *Shigella* and diarrhoea cases

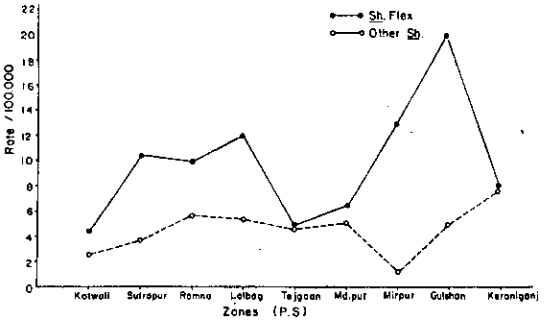

Fig. 2: Zonal distribution of shigellosis cases treated in the ICDDR,B hospital (rates per 100,000 population) in 1980

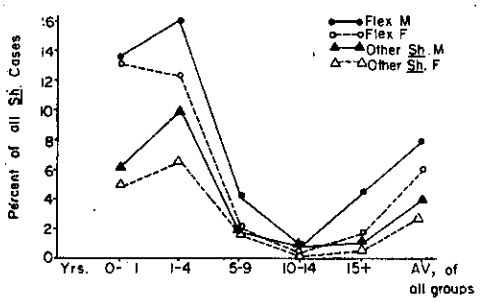

Fig. 3: Age and sex distribution of *Shigella* cases treated in ICDDR,B Hospital

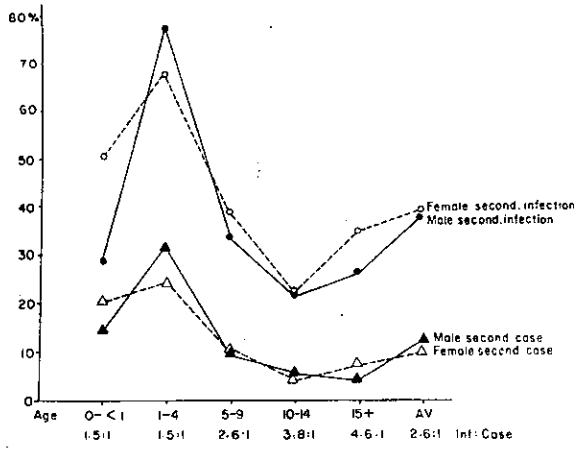

Fig. 4: Secondary infection and case rates by age and sex

The secondary infection and case rates were also higher for children aged up to 9 than for 10 and over. The total secondary infection and case rates were 27.3% and 10.6%. The infection-to-case ratio decreased with the increase in age.

Figure 5 shows incidence of *shigellae* by serotypes. The secondary infection and case rates of *S.flexneri* were 32.3% and 12.8%. The infection and case rates of *S.dysenteriae* were 8.2% and 3.5%. Over 6% had shigella other

than the index types. In the past, *S. dysenteriae* had identical infection rates as *S. flexneri*, but higher secondary case rates than *S. flexneri*.

Figure 6 shows the period of shigella excretion from the date of hospitalization. Excretion for inapparent infection was 1-10 days, hospitalized cases 1-12 days and for non-hospitalized cases 1-9 days. The average for hospitalized cases was 4.2, non-hospitalized cases 4.0, and inapparent infection 3.3 days. More than 25% excreted for one day and 60% excreted for 3 days.

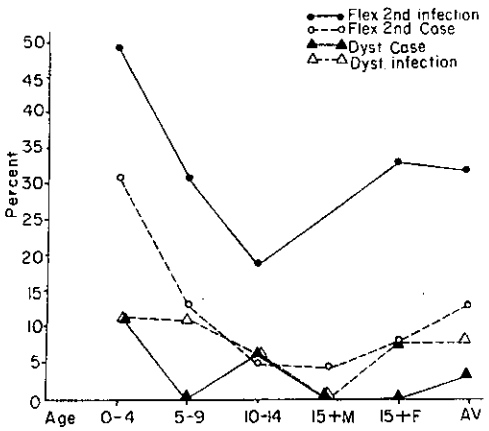

Fig. 5: Infection and case rates of shigellosis by serotypes and age of contacts in percent

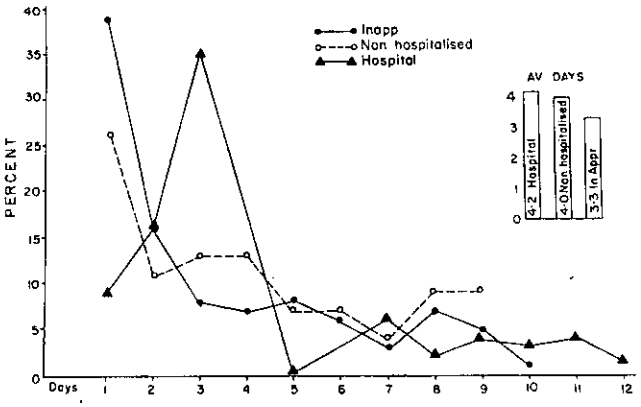

Fig. 6: Duration of excretion of *Shigella* in days and by types of cases in percent

Figure 7 shows the duration of illness. Hospitalized cases suffered from 1- to over 11 days from onset, and non-hospitalized cases 1-10 days from onset. The average for hospitalized cases was 5.7 and for non-hospitalized cases 3.4 days. The illness for *S. flexneri* was 4.8 and for *S. dysenteriae* 5.7 days, on the average.

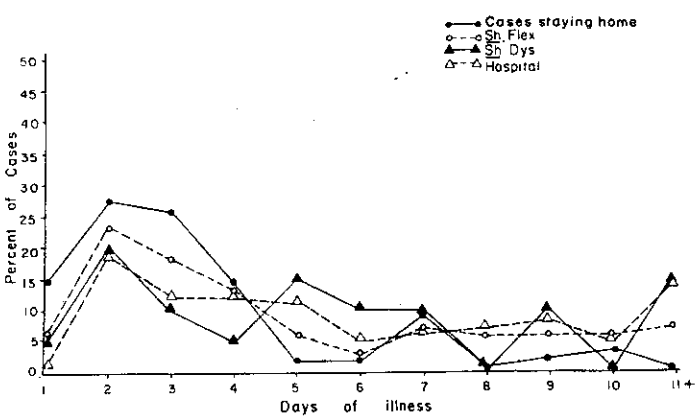

Fig. 7: Duration of illness by types of cases and days of illness in percent

Table 1 shows the relation between crowding and infection rates. The rates were not different between families having 1-3 rooms. Families having more than 3 rooms had no secondary infection. Persons-per-room also did not seem to affect the infection and case rates.

TABLE 1

CROWDING AND SECONDARY INFECTION RATES

Rooms per family	Total contacts	Person/ room	Infection rates No.	%	Case No.	rates %
1	165	5	43	26.0	13	7.9
2	256	3	79	30.8	34	13.3
3	73	2	19	26.0	8	11.0
4	13	3	-	-	-	-
5	10	2	-	-	-	-

Difference in infection rates between families with one room and three rooms = N.S.

Difference in infection rates between families up to three and more than three rooms is significant P=<.01.

Table 2 shows the types of water used and rates of infection. Though the secondary infection and case rates were 60% and 20% in people using open water sources, compared to 28% and 11% in users of closed sources, it was not, however, statistically significant. This was possibly due to small numbers of cases. In a previous study in Dhaka this was a significant association.

The use of open and closed latrines and the infection rates are shown in Table 3. Although users of surface latrines had higher secondary infection and case rates, the differences were not significant. In the past, the difference was found to be significant.

Table 4 shows that 8 (3%) out of the 264 *Shigella flexneri*, excreted by same patients, changed their sensitivity pattern within a few days. It is seen that 1.5% developed resistance against Tetracycline, 0.8% against Chloramphenicol, 1.9% against Ampicillin and 1.1% against Kanamycin.

DISCUSSION

The seasonal incidence of *shigellae* is different from cholera and ETEC diarrhoea in Dhaka. It is low during autumn and winter. The peaks may be related with spring showers, as has been reported elsewhere, and with

TABLE 2

SOURCES OF WATER AND SECONDARY INFECTION RATES

Sources	No. of Contacts	2nd No.	Infection %	2nd No.	Cases %
City supply + hand pump tubewell	388	110	28.3	44	11.3
Tap, tubewell pond + river + canal	124	28	22.6	10	8.0
Open sources canal + river+ pond	5	3	60.0	1	20.0
Total	517	141	27.3	55	10.6

Difference between closed and mixed sources users = N.S.

Difference between closed and open sources users = N.S.

TABLE 3

TYPES OF LATRINE USED AND SECONDARY INFECTION RATES

Types of Latrines Used	No. of Contacts	2nd No.	Infection %	2nd No.	Cases %
Surface, open pit, service	249	77	28.5	30	12.0
Sanitary	268	70	26.1	25	9.3

Differences in 2nd infections and cases are not significant (N.S.)

TABLE 4

PATTERN OF CHANGE OF SENSITIVITY OF *SHIGELLA FLEXNERI* FROM THE SAME INDIVIDUALS OBTAINED IN 10 DAYS

	First Test, Sensitive to					
Test	T	C	A	Sep	G	K
First	4	8	8	8	8	8
Second	0	6	3	8	8	5
Developed resistance	4	2	5	0	0	3
Percent of change of resistant	1.5	0.8	1.9	0	0	1.1

fly breeding. The highest incidence in Gulshan zone is possibly due to the closeness of the hospital to people, resulting in greater utilization isolation from mild cases. Thickly populated areas of old the city have more cases. All *Shigella* are more common in children aged less than 5 years. Males have a higher hospital attendance, possibly due to a larger male population in the city. Secondary infection and case rates are higher in contacts aged less than 5. This may be due to their frequent contacts with peer groups, parents, and family members. This may be also due to poor resistance and poor hygienic practices compared to older ones. The lower secondary infection and case rates of *S.dysenteriae* type 1 than in the past may be due to development of resistance to the single type, thanks to endemicity for some years. *S.flexneri* having many serotypes has more scope to infect people, and hence there are higher secondary infection and case rates. The duration of excretion is important for secondary infection and for persistence of infection in communities. The average duration of symptom-free infection is over 3 days. As the number of *shigellae* needed for establishing infection is few it can thrive well in a population with poor hygienic practices. The use of surface water, fewer numbers of rooms and the existence of many contacts aged below five years are important factors for its spread and prevalence. Some *shigellae*, especially *S.flexneri*, can, however, change its sensitivity pattern while in the same patient.

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Chapter 8

Is *Shigella* a Problem in Infants? A Comment on Proportional Rates

M. Moshaddeque Hossain, Roger I. Glass and K.M. Belayet Hossain

International Centre for Diarrhoeal Disease Research, Bangladesh,Dhaka, Bangladesh

ABSTRACT

The specific population rates of hospitalization for *Shigella* in Matlab thana were examined to see how they compared with rates of *Shigella* isolation at Matlab hospital. In the hospital, *Shigella* was least frequently isolated from infant patients (39 per 1,000 per year), whereas, in the community, using population-based rates of hospitalization, it was found that infants were more commonly hospitalized than any other age group (57 per 10,000 per year). To examine this relationship further, the deaths attributed to dysentery from the Matlab census were studied, and the age-specific proportionate mortality was compared with age-specific population-based mortality. The data suggested that the problem of *Shigella* hospitalization and dysentery deaths in the infants may be underestimated for a community if proportional statistics are used, because the denominators are inflated by other events, such as diarrhoea episodes or deaths from other causes.

In many hospital-based studies of *Shigella*, a rate of isolation (i.e. the proportion of patients who have *Shigella*) is calculated for patients of different ages. In these studies, infants under 1 or 2 years are among the lowest in rates of isolation. One might conclude that *Shigella* is not a problem in this age group, and can speculate that these infants may be protected by decreased exposure or immunity passed on from the mother's breast milk. In infants, diarrhoeal rates are high and a hospital-based

denominator is inflated by many patients having diarrhoea due to rotavirus or toxigenic *E.coli*, which are particularly common at this age. Therefore, we have examined the specific population-based rates of hospitalization for *Shigella* in Matlab Thana (Number of *Shigella* hospitalizations per 1,000 Matlab residents per year) to see how they compare with rates of *Shigella* isolation at Matlab hospital (No. of patients hospitalized with *Shigella*/ total number of patients hospitalized per year).

In Matlab hospital, *Shigella* is least frequently isolated from infant patients (Table 1) (39 isolations/1,000 patients/year). However, in the community, using population-based rates of hospitalization, infants are more commonly hospitalized than any other age group except weanling children (57 hospitalizations/10,000 people/year).

TABLE 1

COMPARISON OF SHIGELLA ISOLATION RATES USING HOSPITAL-VS POPULATION-BASED DENOMINATORS, MATLAB, 1978-1980

	Denominator	
Age	Hospital Cases/10^3 Patients/Yr	Population Cases/10^4 People/Yr
<1	39	57
1-4	106	78
5-9	86	15
10-14	59	4
15-29	53	7
30-44	49	7
45-59	91	7
60+	87	
Total	75	30

To examine this relationship further, we looked at deaths attributed to dysentery from the Matlab census volumes for 1978 (Table 2). 312 dysentery deaths accounted for 14 percent of all deaths in the area (Table 3). We compared age-specific proportionate mortality (Number of dysentery deaths/ total deaths per year) with age-specific population-based mortality (Number of dysentery deaths/total population at risk per year). Using a proportionate

TABLE 2

DEATHS FROM DIARRHOEAL DISEASES, MATLAB, 1978*

Age	Total	Diarrhoea		Dysentery	
		Acute	Chronic	Acute	Chronic
1	728	18	10	8	8
1-4	493	18	14	67	78
5-9	89	1	1	10	7
10-14	40	2	1	1	2
15-29	83	5	0	0	1
30-44	96	1	0	1	5
45-59	198	1	3	6	27
60+	555	4	3	27	64

* From Demographic Surveillance System-Matlab, volume 7.

TABLE 3

DYSENTERY MORTALITY RATES

Age	Proportionate: Dys Deaths x 100 / Total	Population: Dys Deaths x 10^4 / People
1	2	26
1-4	29	66
5-9	19	7
10-14	7	1
15-29	1	1
30-44	6	2
45-59	17	20
60+	16	94
Total	17	19

mortality, we find that relatively few infants die of dysentery (<2%). Nevertheless, since mortality in this age group is so high for other reasons, the population-based death rate for dysentery among infants is very high (26 dysentery deaths/10,000 people/year), and second only to the rate for weanling children.

These data from Matlab suggest that the problem of *Shigella* hospitalization and dysentery deaths in the infant age group may be underestimated for a community if proportional statistics are used. This occurs because the denominators are inflated with other events, such as diarrhoea episodes or deaths from other causes in the age group being examined.

Discussions

Dr. Kostrezewski

As a matter of fact, we know the changing pattern of dysentery in one part of the world, in the country which enjoys much better economical situation. On the other hand, we know very little about dysentery in many other countries. As a matter of fact, only recently, maybe for the last 10 years, can we speak about the entire South-East Asian Region. We have some information from Vietnam, and we know that it is a big problem there. We have a lot of information from here in Bangladesh. And, based on the information from China, I will say the situation there seems to be slightly different. I think that here in Bangladesh in certain ways, we are working in a complex situation from the epidemiological point of view. I would say the modern field laboratory at Teknaf is extremely good for epidemiological type work. Now, my question is, as Dr. Sutton has mentioned, "Why should we look for the appropriate medium for the *Salmonella* group and the *Shigella* group?" I agree that we should look, but not necessarily, for one medium.

Dr. Levine

One possibility: *flexneri* and *dysenteriae* do not survive in the environment as well as the *sonnei*, which is hardy. To transmit *dysenteriae* you have to have strikingly poor hygiene, typical of primitive, less developed areas and certain custodial institutions. In the U.S.A., for example, in institutions that take care of severely retarded children, they have *flexneri* 2a. In training institutions they have *sonnei*. Improvements in hygiene might explain why *flexneri* disappears, but do not explain why *sonnei* takes its place. Epidemiologically, *sonnei* is not a measure of development. In the U.S.A., *sonnei* appeared in day-care centres and nurseries around the country. A similar phenomenon occurred in the Scandinavian countries. One hypothesis is that *sonnei* is spread by children, but the mechanism is different from that of *flexneri* or *dysenteriae*. My own hypothesis is that the spreading mechanism involves toilet areas. It is seen that when children return to schools in September, *sonnei* has the highest prevalence.

Chapter 9

Problems in Bacteriological Diagnosis of Shigellosis

Hanna Stypułkowska-Misiurewicz

National Shigella Centre, State Institute of Hygiene, Warsaw, Poland.

ABSTRACT

Laboratory methods used for the bacteriological diagnosis of shigellosis are much less sensitive than for salmonellosis. *Shigellae* having the ability to penetrate epithelial cells, to reach submucosa and to multiply there, are less numerous in stools than other intestinal bacteria. They survive less well than do *Salmonella* in the faeces, particularly at high atmospheric temperatures. The most pathogenic *Shigella* toxins are most sensitive to the presence or absence of some components used in bacteriological media. Recently, some steps in bacteriological procedures have been rechecked and modifications proposed. Generally, proposed methods are more sensitive in experimental conditions, but less in routine practice of laboratory work. Bacteriological examination is still human dependant, regarding collection of specimens and isolation procedures. So, the human factor is the keystone of success in laboratory investigation, as well as proper collection of samples.

Epidemiological investigations and surveillance of infectious diseases are essential to any control programme. The success of a surveillance programme will depend on the reporting of cases in a community and the investigation of cases, especially those which are clustered. Three essential factors influence reporting: attitudes of the health authorities, availability of treatment, and efficiency of laboratory services concerned. Cases

may be missed if physicians do not have or do not use laboratory facilities. As far as dysentery is concerned, laboratory diagnosis may be decisive for clinical management of patients, and it is essential for surveillance.

There are three points in bacteriological diagnosis of shigellosis that still need further developments. First, elaboration of better methods for transportation of faecal specimens, capable of preserving *Shigella* for a long time. Second, development of proper selective enrichment media and selective differentiating media for isolation of Shigella. Third, but perhaps the most important, elaboration of effective and simple methods for quick diagnosis of *Shigella* infections, especially in field conditions.

This conclusion comes from the review that follows.

The direction that faeces from dysentery patients should be inoculated immediately at the patient's bed, may be followed only rarely. Usually the specimen is transported to the laboratory, which sometimes is located at a distance. This influences the results significantly. The mode of collecting the specimen is important too. The problem now is which is better: plain faecal samples or faecal swabs? Swabs are easy to handle. They can be taken immediately, easily transported or stretched on media at once. Some authors consider swabs equivalent to stool specimens. But summary of routine investigations carried out in Poland has shown that more positive results for *Salmonella* and *Shigella* are obtained by various workers from stool specimens than from swabs (Table 1). The differences may be explained by the risk that swabs may not contain the faecal material. Under the circumstance, the risk may be as high as up to 30-50%. Swab samples may be taken directly from *Shigella* ulceration through rectoromanoscopy. In those cases the positive results may be superior to those obtained from plain stool samples (1,2).

As far as plain faecal samples are concerned, in order to isolate *Shigella* from acute cases of dysentery, the rule that great care has to be exercised to use washed fragments of mucous for plating still remains valid.

If any significant delay in reaching the laboratory is unavoidable, the use of a transportation medium is indicated. *Shigella* do not survive as well as *Salmonella* or *Escherichia coli* in the faecal samples, particularly at high atmospheric temperatures. They are sensitive to metabolic fermentation products of other components of the bacterial intestinal flora, as well as to phages, colicines etc.

Efficiency of various media used to transport faecal specimens for isolation of *shigellae* has been evaluated by Morris and co-workers (Table 2, 3). Compared to direct plating, the best results have been obtained with buffered glycerol saline, which preserved *Shigella* at least twice as well as other transport media. Anyway, the buffered glycerol saline is generally used both for preservation of faeces samples and faecal swabs. The filter-paper method that has been used during war-time in field conditions, is less satisfactory for *shigellae* than for *salmonellae* (4,5). We know that some territorial laboratories have switched to this method, which resulted in a

TABLE 1

SUMMARY OF ROUTINE BACTERIOLOGICAL EXAMINATIONS CARRIED OUT IN POLAND IN 1978, PERCENTAGE OF SPECIMENS FOUND SHIGELLA - OR SALMONELLA-POSITIVE, DEPENDING ON THE TYPE OF SPECIMEN AND ITS ORIGIN (FROM THE ANNUAL REPORT OF THE PUBLIC HEALTH LABORATORIES)

Origin of the specimen	Type of specimen					
	Stool samples			Swabs		
	Number examined	Positive		Number examined	Positive	
		Number	%		Number	%
Patients	259378	22073	8.5	257466	13853	5.4
Convalescents	22585	2673	11.8	20455	1782	8.7
Carriers	25556	4362	17.1	15805	2336	14.8
Contacts	128393	3216	2.6	124273	1638	1.3
Food handlers	2138684	6036	0.3	6087790	1014	0.1

TABLE 2

EFFICIENCY OF VARIOUS MEDIA USED TO TRANSPORT FAECAL SPECIMENS FOR THE ISOLATION OF *SHIGELLAE* (3)

Transport or enrichment media	Days media held before plating	No. faecal specimens examined	No. *Shigella* isolated by direct plating of faecal specimen	No. *Shigella* isolated when specimens plated from transport media	Percent positive compared to direct plating
Buffered-	1-2	36	18	15	83.0
glycerol-	3-6	271	50	24	48.0
-saline	7-10	203	45	17	37.8
Cary-Blair	1-2	298	68	18	27.5
	3-6	148	35	8	22.8
	7-10	295	27	8	29.6
GN broth	1-2	298	66	8	12.1
	3-6	125	28	2	7.1
	7-10	24	8	0	0
Silica gel	3	18	8	3	38.0
SP broth	7-10	148	14	1	7.1

dramatic reduction in the frequency of *Shigella* isolations thereby giving an impression that "the epidemiological situation of shigellosis has improved".

Because of generally unknown quantity of *Shigella* in stool samples, cultures should be made onto at least two plates; one non-selective (MacConkey or EMB) medium and one selective (desoxycholate citrate agar or so-called SS) medium. According to some authors, both the selective media are

TABLE 3

COMPARISON OF FIVE PLATING MEDIA FOR THE GROWTH OF DIFFERENT *SHIGELLA* SEROTYPES (MEDIAN RESULTS FROM INOCULATION OF THREE DIFFERENT STRAINS OF THE SAME SEROTYPE)/9,10/

Shigella serotypes		Decimal logarithm of the number of bacterial colonies grown on:				
		Nutrient agar	EMB	SS (Difco)	SS (WSS-1970)	SS (with oxbile)
S.dysenteriae	2	4	3	3	2	3
S.flexneri	1a	4	4	3	O	3
	1b	4	4	3	2	3
	2a	4	3	3	2	3
	2b	4	3	3	2	3
	3a	4	3	3	3	3
	3b	4	4	O	O	O
	4a	4	4	3	/1/	1
	4b	4	4	4	3	4
	5	4	4	4	3	3
	6	4	4	/3/	/2/	/2/
	X	4	3	/2/	O	O
	Y	4	4	3	/2/	3
S.boydii	1	4	3	3	2	2
	4	4	3	1	O	2
	7	4	3	O	2	1
S.sonnei		4	4	2	2	•

O-lack of growth after 48 hours
/ / - growth after 48 hours
• not examined

superior to MacConkey medium, especially for examination of convalescents and contents. For some strains, however, they may be too selective, especially for *S.dysenteriae* (6,7,8) and some *S.flexneri* and *S.boydii* serotypes (Table 3) (9,10). Recently described Hektoen enteric (HE) medium and cylose lysine deoxycholate (XLD) medium did not seem to be better (11). Brilliant green media, including Wilson and Blair's medium used in bacteriological diagnosis of salmonellosis, are not suitable for the growth of *Shigella*.

There are no good enrichment media for *Shigella*. Tetrathionate medium used for *Salmonella* is unsuitable for *Shigella*. *S.sonnei* and some serotypes of *S.flexneri* (6, 3a) may survive in some lots of the selenite broth, but other *Shigella* serotypes do not. This may depend on the quality of peptone and proportion of phosphates in the medium (12). The Hajna's GN broth (13), and other enrichment media (14) are not sufficiently selective, the growth of *Escherichia coli* is suppressed only for a rather short time (4-5 hours), and that of Proteus not at all.

Nevertheless, it may happen that some *Shigella* strains may be isolated after enrichment in those media, although direct plating gave no positive results. More than a thousand specimens from diarrhoea cases in Warsaw were examined using two selective media (MacConkey and SS) and three enrichment media (SF, SH I and SH II). It was found that SH I and SH II are rather similar in performance but differ from GN broth (Table 4). Eighty-two positive results

TABLE 4

COMPOSITION OF THREE *SHIGELLA* ENRICHMENT MEDIA

	GN broth (13)	SH-I (15)	SH-II (15)
Peptone	20 gms	10 gms	10 gms
Beef extr.	-	-	5 gms
Glucose	1 gms	-	-
Mannitol	2 gms	5 gms	10 gms
Sodium citr.	5 gms	20 gms	20 gms
Sodium thiosulf.	-	15 gms	8.5 gms
Ox bile	-	400 ml	8.5 gms
Sodium desoxychl.	0.5 gms	-	5.0 gms
Dist. water	1000 ml	1000 ml	1000 ml

were obtained, of which forty-two were positive only after enrichment, but no separate medium could be omitted without reduction of positive results (Table 5). Even comparatively inefficient SF medium increased the number of

TABLE 5

EFFICIENCY OF VARIOUS MEDIA USED TO INOCULATE FAECAL SPECIMENS FOR THE ISOLATION OF *SHIGELLAE* (15)

Plating and enrichment media	Number of *Shigella*-positive specimen		
	found by inoculation of the medium		found negative on the other media
	Number	Per cent	Number
Direct plating (MC+SS)	40	48.8	8
Indirect plating (MC+SS) after enrichment on			
SH I	52	63.4	9
SH II	47	57.3	2
SH I + SH II	57	69.5	15
SF	34	41.5	8
SH I + SH II + SF	74	90.2	42
All together MC+SS+SH I + SH II + SF	82	100.0	82

positive results by nearly ten percent (15). This may be due to various factors. When the number of organisms in the specimen is scanty, the chance factor may play a role. Besides, the amount of the specimen given to an enrichment medium is usually greater than that used for plating. One cannot exclude the possibility of specific qualities of the organisms, both looked for and accompanying, which may cause their greater resistance to chemical components of particular media.

When the use of enrichment and selective media is impossible, the non-selective media may appear quite useful under the following circumstances: if the cases are acute, in the early stage of the disease and untreated. The specimens of course should be taken and plated according to the rules.

Selective media from various manufacturers may differ in their nutritional and selective characters; sometimes there may be difference between lots from the same producer. It is advisable not to forget to control the media for their nutritional, differential and selective qualities. This can be performed using well-selected control strains. The strains should be inoculated onto a selective and - for comparison - onto a non-selective medium. For *Shigella* and *Salmonella* strains, the number of colonies on the selective medium and the non-selective one should be similar. For *Escherichia coli* strains, a count of about 100 times lower on the selective medium than that on the non-selective proves to be a good selective quality of the medium concerned (16) (Figure 1).

The quality of an enrichment medium may be checked by measuring the density of the culture, and by counting the number of living bacterial cells in 1 ml of the culture grown from a well-known inoculum (16).

The newly-proposed selective or enrichment media should be checked by a number of diagnostic laboratories in their routine work, and evaluated in comparison with the results obtained, in parallel, in their usual way of examination. A study by W.I. Taylor and D. Schelhardt is a good example for such a trial (11). However, their specimens contained *S.sonnei* only. It would be desirable to see the results of other laboratories whose material contains a much broader spectrum of *Shigella* serotypes.

Many workers carry out preliminary *Shigella* identification by slide agglutination from the bacterial growth on the primary plates. It is more expensive than the biochemical identification and often misleading too. In any event full biochemical testing should be performed with subcultures that have been checked for purity. The Taxonomical Committee of IAMS (17) has decided that *Shigella* classification into species and sub-species is based only on the biochemical characters, and the antigenic structure is used for sub-division into serological types, sub-types, and variants.

How many biochemical tests should be used for diagnostic purposes depends on the capability of the laboratory. This, however, may influence the effectiveness of the laboratory work. Evaluation of two methods of biochemical determination and differentiation of *Shigella* and *Salmonella*

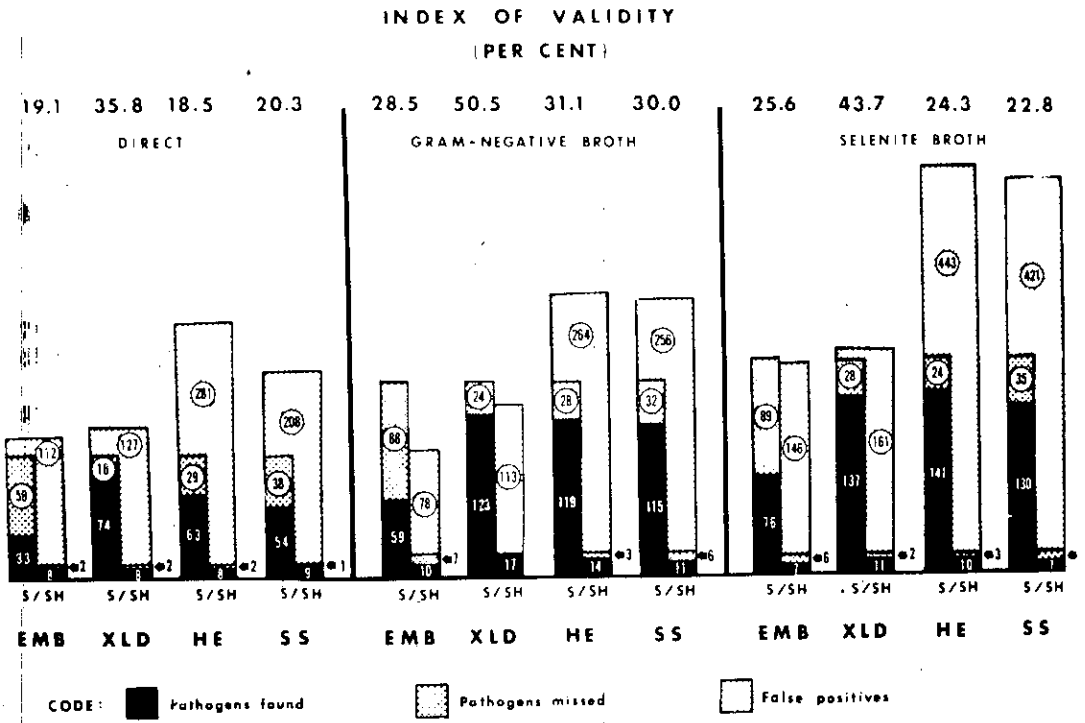

Fig. 1: Comparison of four plating media for the isolation of *Salmonella* (S) and *Shigella* (SH) from 1,597 stools. Abbreviations: EMB, cosin methylene blue; XLD, xylose lysine deoxycholate; HE, Hektoen enteric; SS, *Salmonella*, *Shigella*.

strains, used in Public Health Laboratories in Poland was carried out by Macierewicz (18). The addition of 7 tests supplementary to the formerly used set of three differentiation media (urea medium, peptone water, and Kligler medium) at the routine examination of 7064 isolated colonies allowed to reduce the number of false results (1 false positive and 5 false negatives instead of 7 and 47 respectively). Besides, the number of slide agglutinations to be carried out was reduced significantly.

The development of various quick biochemical tests available commercially, may enable the use of a greater number of biochemical tests, as well as getting results in a shorter time. However, besides the growing cost of those tests, there is a question of some margin of false results, however narrow these might be. This direction of development is, however, very desirable, particularly when the needs of field examinations are concerned (19,20,21).

As far as a detailed serological determination of *Shigella* strains is concerned, there are no problems if good diagnostic sera are available. In some countries there are *Shigella* reference laboratories which are helpful in checking the correctness of diagnostic activities of other laboratories. At

present, there is no evident sign of any activity of the International *Shigella* Centre. There seems to be a lack of activity in the international collaboration on *Shigella* problems.

There is a need for a simple and rapid method of laboratory diagnosis of shigellosis, suitable for field investigations, something like Bebebson's method for *Vibrio cholerae* identification (22). This might be useful in cases of diarrhoeal epidemics, particularly in developing countries or remote regions where laboratory facilities are very often unavailable.

The frequency of *Shigella* isolation from dysentery cases differs depending on various conditions and clinical and laboratory experience. In Poland, isolation rates from clinical hospitalised cases amount to 60-70%; whereas from dispensary patients during the communal water-borne outbreaks, only to 25-27% of suspected cases. Many reasons may be responsible for that: there may be a difference in the technique of sampling and in transport of the samples, difference in the work level of the laboratories concerned, a difference in the variety of aetiological agents responsible for dysentery symptoms. Often it is difficult to know which of those factors plays a role, and to what extent. For example, the surveillance of *Shigella* infections in Poland showed the difference in frequency of infections in neighbouring regions (23). It is difficult to determine if there were really different ecological conditions, or maybe the differences in registration, and bacteriological investigation of specimens due to differences in the competence of the staff. They were supposed to follow the unified diagnostic methods (24).

Thus, many problems of bacteriological diagnosis of shigellosis are waiting for solution and development. The possibility of the aetiological role other than *Shigella* micro-organisms themselves, even in most typical cases of dysentery, complicates the tasks, but makes them at once more attractive (25).

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Discussion

Dr. Kostrezewski

I fully agree with Dr. Mata, that it is a case of better hygiene. However, I am going to ask more questions. I would like to ask why we have *Shigella sonnei* at the same level or at an increasing level compared to *S. flexneri?* We had sporadic imported cases of *Shigella dysenteriae* type 1, *Shigella boydii, Shigella flexneri* etc. You said it has come from Egypt, from Alexandria. We examined Dr. Hanna's summary, which said that about 60% of them after one year and about 8% of people are still excreting these. But these were not spread around the community. They were isolated, sporadic cases, which continue discharging the bacteria, but don't infect others. In one family we had a big epidemic of *S.sonnei.* Every year we still have 9,000-to-10,000 cases of *Shigella sonnei,* mainly in children. So, my question is, "What hygiene can work against this seasonality and improve the destructive situation; and why does hygiene not seem to offer protection against *S.sonnei?"*

Dr. Rahaman

Since 20 percent of diarrhoea episodes still go undiagnosed, new pathogens, such as *Campylobacter* or *Yersinia interocolitica* should be sought. Continued attention should be paid to work on new media, which can better isolate *Shigella,* as well as other important pathogens.

Dr. K.M.S. Aziz

If necessary, as Dr. Hanna indicated, we can have certain definite media for *Shigella flexneri.*

Chapter 10

Surveillance of Shigellosis in Matlab: A Five Year Review

K.M. Belayet Hossain, Roger I. Glass, M. Imdadul Huq, Md. Yunus and M. Moshaddeque Hossain

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

ABSTRACT

Since 1977, stool samples of all patients from a defined surveillance area attending the field hospital of the ICDDR,B in Matlab Bazaar have been cultured for a variety of pathogens including *Shigella*. We have reviewed the seasonality of isolation, and the age distribution of patients by *Shigella* species, and have examined the pattern of antibiotic resistance for selected isolates. Of the 30,855 patients examined between 1977-1980, 1,978 (5.7%) had *Shigella*, making it the third most commonly isolated enteric pathogen after enterotoxigenic *E.coli* (ETEC) and rotavirus. *S. flexneri* comprised 59% of these isolates, followed by *S.boydii* (16%), *S. shiga* (12%), *S.sonnei* (8%), *S.schmitz* (4%) and *S.dysenteriae* types 3-10 (1%). *Shigella* was less frequent in the winter and the monsoon seasons, consistent with the hypothesis that it is a water-washed disease but does not show the marked seasonality of *V.cholerae*, rotavirus or ETEC. Children and the elderly are affected more often than adults, and there seems to be little difference in these attack rates by *Shigella* species. Occasional multiple antibiotic-resistant strains of *Shigella* have been isolated although they never occurred in significant numbers. *Shigella* is a pathogen of major importance in rural Bangladesh, that deserves more attention from the perspective of diarrhoeal disease control in the community.

INTRODUCTION

Since 1963, the ICDDR,B has operated a rural treatment centre for diarrhoeal diseases in Matlab Bazaar. This area, 45 km southeast of Dhaka, occupies a portion of the delta that is intersected by numerous rivers and canals. The area is very fertile and among the most densely populated in the world. Every September, after the monsoon rains, the river level rises 14 feet above its lowest point, submerging about one-third of the available land.

The treatment centre has maintained a microbiology laboratory to support patient care and epidemiologic studies. Before 1978, patients with diarrhoea were screened for *V.cholerae*, *Salmonella*, *Shigella* and amoeba. A pathogen could be identified in only about 25 percent of the patients. Since 1978, techniques for isolating enterotoxigenic *E.coli* and rotavirus have been included, and a diagnosis for about 75 percent of the 4,000 patients seen each year can be made. Since 1978, only patients from a defined surveillance area have been investigated.

We have reviewed microbiology reports of patients who attended the Matlab treatment centre since 1976 to determine the seasonality of *Shigella* and the age-specific rates of isolation.

RESULTS

Between January 1, 1978 and December 31, 1980, 14,127 patients from the surveillance area were admitted to the Matlab hospital and cultured for enteric pathogens. *Shigella* has been isolated from 7.5 percent of these patients, ranking it fourth after enterotoxigenic *E.coli*, rotavirus, and *V.cholerae*. *S.flexneri* is the most common species, followed by *S.dysenteriae* type 1, *S.boydii*, *S.sonnei*, and *S.dysenteriae* type 2. Isolation of *S. dysenteriae* types 3-10 were rare (Table 1).

Shigella was most commonly isolated from weanling children aged 1-to-4 years and the elderly people aged 45 years or more, and is very rarely isolated from infants less than 1 year. These infants have the largest total number of diarrhoeal events with *E.coli* and rotavirus, so the rate of *Shigella* isolation in this group is, in part, an artifact of the denominator.

The seasonality of *Shigella* is marked by a large peak in the dry season after the monsoon (September to December) and a lull during the cold winter (February) (Figure 1). This reflects the pattern of *S.flexneri*, the most common species (56%) and is similar to the seasonality of *S.dysenteriae* and *S.boydii* although their numbers are small (Figure 2). There is considerable variation in the total number of isolates from year-to-year, but there has been a steady decline between 1976 and 1981.

TABLE 1

RATES OF *SHIGELLA* ISOLATION BY AGE AMONG MATLAB HOSPITAL PATIENTS, 1978 - 1980

Age	1978-80 Total Patients	*SHIGELLA* Species-Rate/1,000/Yr					
		S. flexneri	*S. boydii*	*S. dysenteriae* I	*S. dysenteriae* II	*S. sonnei*	*Shigella* Total
< 1	2780	27	5	1	1	4	39
1 - 4	4880	65	11	15	4	10	106
5 - 9	1333	41	11	22	5	6	86
10 - 14	695	27	6	14	6	6	56
15 - 29	1982	31	9	8	3	2	53
30 - 44	1092	24	13	9	2	1	49
45 - 59	373	30	35	11	3	11	91
60 +	992	57	9	15	2	2	87
Total	14127	44	10	12	3	6	75

A sample of the *Shigella* isolates has been tested for their antibiotic resistance, as part of the treatment trial (Table 2). Most are sensitive to ampicillin, chloramphenicol, kanamycin, gentamicin, and trimethoprim-sulfamethoxazole. Most recently, we have begun to isolate multiple-resistant *Shigella* demonstrating resistance to tetracycline, ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol.

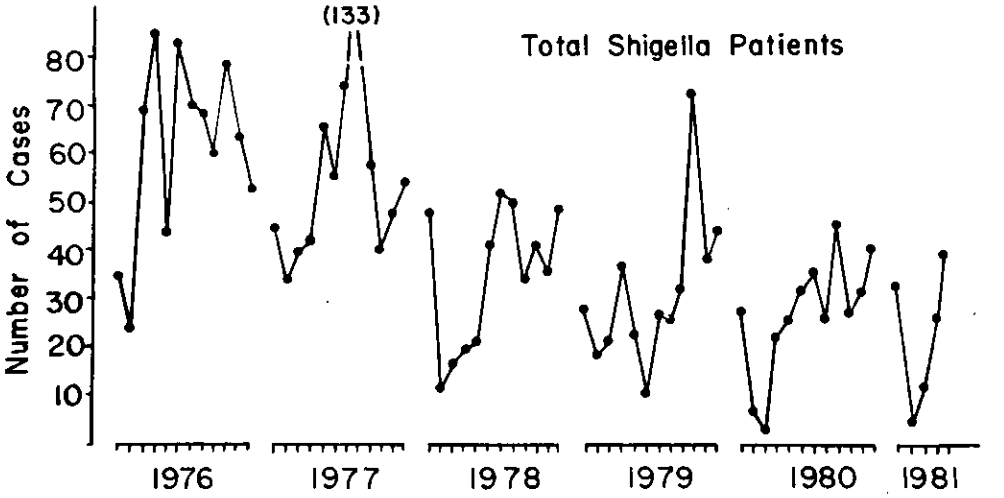


Fig. 1: *Shigella* surveillance at Matlab Hospital, Bangladesh, 1976-1981

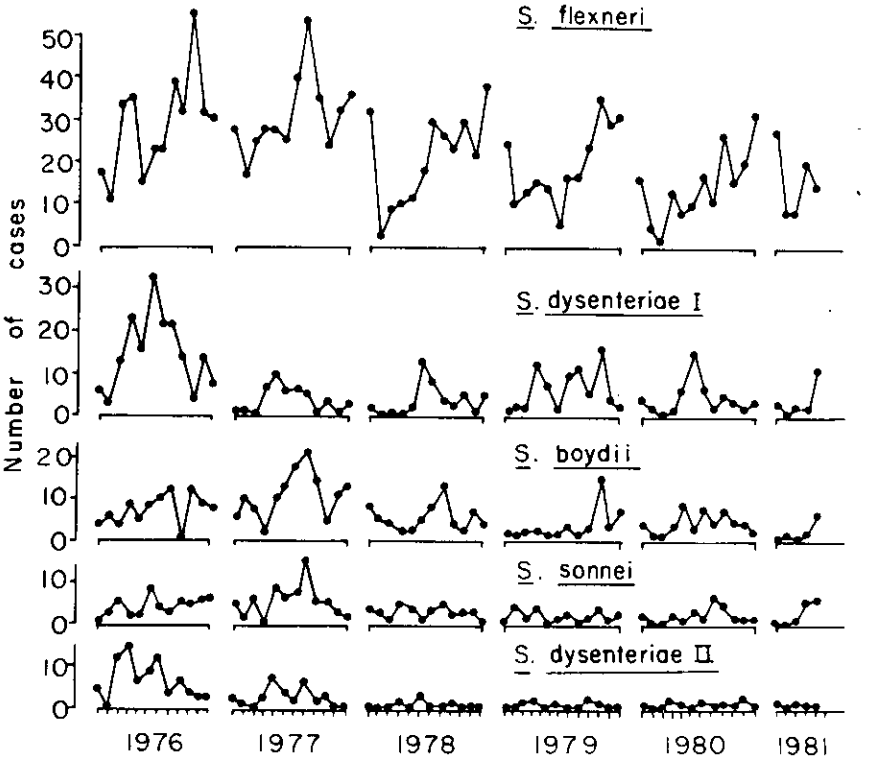


Fig. 2: *Shigella* surveillance at Matlab Hospital, Bangladesh, 1976-1981

TABLE 2

ANTIBIOTIC RESISTANCE PATTERN OF *SHIGELLA* STRAINS ISOLATED FROM TREATMENT TRIAL

Antibiotics	% of *Shigella*-Resistant			
	Shiga (N=35)	Flex (N=38)	Others (N=5)	Total (N=78)
Tetracycline	93	74	75	93
Ampicillin	5	7	20	7
Chloramphenicol	0	8	0	4
Kanamycin	0	5	0	2
Gentamicin	0	0	0	0
Streptomycin	94	89	80	91
Trimethoprim-sulfamethoxazole	5	0	20	3

Source: M. Yunus *et al* (1981)

COMMENTS

In Matlab, *Shigella* is the fourth most common pathogen currently isolated; 7.5% of all patients with diarrhoea are *Shigella* positive but the rate of isolation has been declining gradually for five years. This rate is lower than hospital or clinic isolation rates observed from the Dhaka Treatment Centre or the Teknaf Dysentery Project Clinic of the ICDDR,B. Matlab is a rural deltaic area with an abundance of water and an ecology unlike the overcrowded urban areas of Dhaka or the dry highlands of Teknaf. These ecological differences may be important in explaining the difference in the overall incidence of the disease. In all these areas, the cold season is associated with the lowest incidence of *Shigella*. In the hospital, *Shigella* could be suspected most often among children aged 1 to 9 years. The absence of significant resistance to ampicillin and trimethoprim-sulfamethoxazolemakes these drugs most useful in the period described.

Discussions

Dr. Rahaman

I want to comment on the seasonality of shigellosis. You will see that in Dhaka, shigellosis has a definite monsoon peak, the same thing was found in Teknaf, both in the community-based studies, and from the isolation of the patients coming to the Centre.

It seems that the monsoons have some role to play in the secondary spread of shigellosis. What would be the mechanism? In monsoon we know there is a lot of water and that is against the hypothesis that *Shigella* is a water-washed disease. In Matlab in the monsoon, there is water all around you, and probably that is one of the reasons why shigellosis is so low: because people will go to the canal, to the tank and to the rivers and have ready access to plenty of water -- to wash their bottom, wash their hands, take a daily bath; and naturally the *Shigella* rate is low. Maybe that is the reason why we have a flood-curve in Matlab.

In Dhaka, it is an urban situation. Teknaf is drier, but during monsoon there is plenty of water. Why should there be such a peak in monsoon?

In Poland, I noticed, there also is an epidemic peak, though there are no monsoon rains. In Poland epidemic peaks probably are due to social mixing, to heavy person-to-person contact during periods of bad weather when people remain indoors. This hypothesis is supported by evidence from all three areas, Teknaf, Matlab and Dhaka -- evidence which shows epidemic peaks during the monsoon season, when people tend to remain indoors for days at a time.

Dr. Khan

I would like to add a post-script to Dr. Rahaman's comments. The peak we find in Dhaka is not really during monsoon but in pre-monsoon and post-monsoon periods. The peak for *Shigella flexneri* occurs in May, the pre-monsoon time and also in October, November, December and January, which is post-monsoon and dry.

Dr. Glass

Seasonality varies from year-to-year. Thus, this year the rainfall began early, but normally it's in July and August. In the period when rainfall was highest, the rate of *Shigella* incidences came down. At the end of the month the rates went up again. The dry season was hotter, and a dip in incidences was seen in the cold winter months.

Dr. Kostrezewski

I wonder how many mothers were prevented from reporting sickness to hospitals because of the monsoon rains?

Dr. Glass

The data is presented as cases per thousand in the hospital, It would affect all mothers. The number of mothers might come down, but the percentage of the distribution of *Shigella* would remain unchanged. These data are presented from a five-and-a-half-year surveillance.

With monsoon, there is a tremendous variability from year-to-year. It's not just a single peak. What we would like to suggest is that it is highest after the monsoon, but the peak in Matlab varies much more than either in Dhaka or Teknaf.

Chapter 11

Bacteriological Diagnosis of *Shigella* in Laboratory and Field Conditions

M.I. Huq, A.K.M.G. Kibriya and M.M. Rahaman

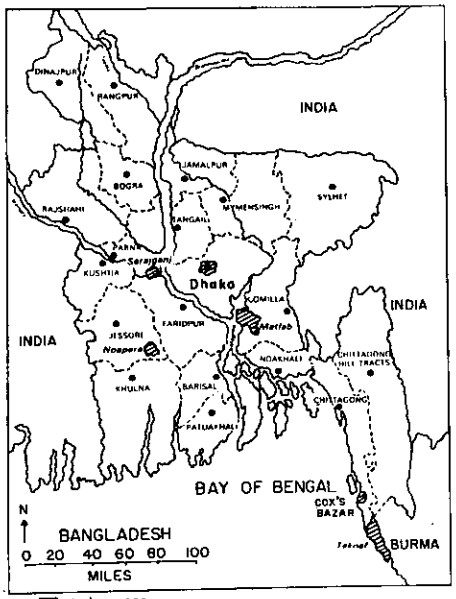
International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

ABSTRACT

Proper laboratory diagnosis of *Shigella* can be achieved in a well equipped Central Laboratory as well as in Field Laboratories, by inoculating the stool or R/S onto specific selective and non-selective plates and incubating the medium in an electric or kerosene-heated incubator to give a temperature range of 29-37°C. When using a lower level of temperature, the time of incubation must be 24 hours or more. Different culture media have been used by different laboratories for the isolation of *Shigella*. The most common ones are MacConkey, SS Agar x LD & HEA. The suspected colonies are confirmed biochemically, followed by serology.

Bacterial enteric infections are a major health problem in developing countries of the world including Bangladesh. Bacillary dysentery due to the *Shigella* group of organisms covers almost 15%-18% of the cases reporting to hospitals for treatment. Accurate laboratory diagnosis and determination of the antibiotic resistance pattern is vital for treatment and surveillance, including the early recognition of epidemics or exotic infections. A simple laboratory procedure for correct diagnosis is a priority for all laboratories dealing with the isolation, identification and characterization of enteric organisms; the *Shigella* group, however, presents special problems.

This paper mainly deals with simple and effective methods for the diagnosis of *Shigella*, from cases or contacts based on the experiences of diagnostic bacteriology laboratories in Dhaka which are well-equipped with modern equipment, and field laboratories in Teknaf which are housed in a semi-pucca building with no electricity and gas connection; and are equipped with kerosene heated incubators, sterilizers and refrigerators (map).



Map of Bangladesh showing Dhaka, Cox's Bazar and Teknaf

COLLECTION AND TRANSPORT OF SPECIMENS

Stool specimens should be collected during the initial or acute stage of diarrhoea, before initiation of chemotherapy. The specimen should be collected as aseptically as possible, and stored in a clean container sterilized or washed properly with boiling water. Disinfectants must not be used for washing. Stool specimens may also be obtained with a catheter and syringe. If a stool specimen cannot be obtained, a sterile rectal swab can be used. The swab must be inserted beyond the anal sphincter and must show faecal staining.

If the faecal specimen (either stool or rectal swab) cannot be processed by the laboratory within two hours, a transport medium should be used. For the isolation of *shigellae*, buffered glycerol saline (1) and Cary-Blair (2) transport medium have been used extensively. Buffered glycerol saline is easy to prepare and has been found more suitable for the isolation of *Shigella*. Cary-Blair medium is suitable for both shigella, *Salmonella* and *Shigella* vibrios. In both cases, the cotton end of the swab is inserted into the bottom of the medium, and the stick is broken off at a level which allows the top to be replaced tightly. If stool specimen is available, a small amount of faeces may be inserted into the transport medium using a stick or glass rod. The specimens should be plated, if possible directly at the bedside or immediately on arrival at the laboratory. If none of these is possible, it should be stored at 4°C. There are several suitable media of varying selectivity for primary plating which a) inhibit the growth of gram-positive bacteria b) inhibit the growth of commensal bacteria and c) differentiate lactose fermenting bacteria (e.g. *E.coli*) from non-lactose fermenting bacteria (e.g. *Salmonella* and *Shigella*.

CHOICE AND HANDLING OF MEDIA

We recommend a minimum set, comprised of at least one selective media and another of high selectivity. Examples of non-selective medium are MacConkey's and EMB Agar. Selective media include DCA, SS Agar, BSA XLD and BGA. All these media are available in dehydrated form, but many of them can be prepared from ingredients locally. Recommended set of media are MacConkey's Agar (3) with any other selective media like DCA (4), SS (5) or XLD (6). We recommend preparation of media in a Central Laboratory equipped with heaters, sterilizers and other essential equipment; but it can be made in a field laboratory also using a kerosene or gas stove and an ordinary high dome pressure cooker. Some medium, such as SS Agar, do not need autoclaving and, as such, media preparation in the field having a small laboratory set up does not pose a problem. The prepared media should be kept in cool places to avoid drying. Morris *et al* (1970) (7) compared several media for direct isolation and transport of *shigellae* from faecal specimens. Their study showed XLD and SS were superior to others, giving 93% and 91% isolations in direct plating. In one of our studies we found the same results. Among 142 isolations from 1212 stool or R/S specimens, (XLD) showed 88% positivity, as against SS 85.5% positivity (8). Later studies have, however, shown that percentage of isolation of drug-resistant *Shigella* from SS Agar was less (9). We also found buffered glycerol saline superior to Cary-Blair Medium for the transport of *shigellae*, but the efficiency of both of these media decreased with time. So preferably these must be plated at an earliest time. However, it has unquestionably been accepted that for the isolation of *Shigella* direct plating of the clinical material is most suitable, as none of the medium tested for enrichment of *Shigella* were found suitable. Further, there is definite evidence that some species of *shigellae* are inhibited by the inhibitory media. For example, *S. dysenteriae* 1 seem to be isolated better from MacConkey's Agar than from SS Agar medium (10).

INOCULATION AND INCUBATION

All the inoculated medium should be incubated at a range of temperature varying from 35°C - 37°C. In the field laboratory where electricity is not available we used a kerosene or bottled gas-heated incubator and found it suitable. The incubator, a small egg hatching type, was heated by a small kerosene lantern, and has been found to give temperatures ranging from 30°C - 37°C. The plates needed to be incubated for at least 24 hours to get proper colonial growth.

RECOGNITION AND TESTING OF ISOLATES

All the suspected non-lactose fermenting colonies on SS or MacConkey and a typical red colony on XLD Agar should be examined biochemically. Two different media which have been tested widely by our laboratory are the combination of Kligler Iron Agar (KIA) and combined Motility Indole Urea Agar (MIU) or Christensons Urea Agar (CU). We found the Kligler iron agar superior to Triple sugar Iron agar in areas where *Vibrio cholerae* and other sucrose-fermenting vibrios are present, as acid reaction due to fermentation of sucrose will make the slant and butt yellow, whereby *Shigella* could be missed. Both sets of medium, either KIA, MIU or KIA, CU, can be inoculated as stab by a straight wire and/or streaking on from the same colony. The KIA demonstrate the fermentation of glucose and lactose, with the formation of acid with or without gas. The test also indicated the ability of an organism to produce H_2S, as shown by blackening along the stab line throughout the tube. The CU agar indicates the ability of an organism to cause hydrolysis of urea, causing red slant. The combined MIU medium shows motility, production of Indole and also hydrolyses urea.

As described earlier, *Shigella* grown on the isolation plates as small-to-medium non-lactose fermenting colonies on MacConkey and SS Agar and small red colonies on XLD Agar, the growth does not always show a defined pattern. We found it advisable to inoculate multiple suspected colonies, if possible, from a single plate. We have observed in our experiments that picking 5 suspected colonies from single samples increases the chances of isolation by 15.6% over picking one single suspected colony (8). So, care must be taken to pick up multiple colonies, if they are suspected.

When the biochemical reactions conform to that of *Shigella* i.e. an acid butt with alkaline slant without H_2S and gas production in KIA and non-motile, Indole variable and urease negative on MIU, the small amount of growth from the slant is suspended in saline and agglutinated with respective *Shigella* antisera. The genus *Shigella* is subdivided into four subgenera or subgroups, according to their biochemical reactions: *S.dysenteriae*, *S.flexneri*, *S.boydii*, and *S.sonnei*. The first three subgroups may be further subdivided by serotyping, but for *S.sonnei* colicine-typing and, less commonly, phage typing is used. There are ten known serotypes of *S.dysenteriae*, eight of *S.flexneri* and fifteen of *S.boydii*. There are also a small number of sub-judice serotypes of *S.dysenteriae* and *S.boydii*. *S.sonnei* has been differentiated into 15 colicin types. Some *Shigella* strains fail to agglutinate in homologous antisera, and this O agglutinibility can be removed by heating a saline suspension in a boiling water bath for 20 minutes. All the antisera are available commercially, but can also be prepared by competent laboratories. On the basis of slide agglutination, a preliminary report can be given which is to be confirmed by full biochemical and serological tests. The sensitivity pattern of the isolate can be determined by putting the antibiotic discs onto a lawn of 4-5 hours grown bacteria on a Muller-Hinton or any commonly used non-selective agar plates by using standard Kirby Bauer method (11).

Table 1 shows the comparative isolation of different pathogens in the Central Laboratory at Dhaka and the Field Laboratory in Teknaf. It may be seen that *Shigella* group of organisms comprises a major portion of the total number of pathogens isolated. The field laboratory, with minimum facilities, was capable of handling a large number of samples, both from clinical cases and supporting field studies.

DISCUSSION

We were able to isolate *Shigella* in a well-equipped Central Laboratory as well as in Field Laboratories by inoculating the stool or R/S onto specific selective and non-selective plates and by incubating the medium in a temperature range of 29°C-37°C in electric or kerosene heated incubators. While using lower level temperature, the time of incubation needed was 24+ hours.

We performed short biochemical tests by inoculating suspected non-lactose fermenting colonies onto KIA and MIU or CU media and on the basis of the reaction pattern cultures confirmed by slide agglutination. Due to emergence of multiple antibiotic-resistant *Shigella* organisms, it is necessary to look at the drug sensitivity pattern of the *Shigella* isolate before therapy is initiated. The drug sensitivity pattern helps the clinicians to initiate proper therapy, as well as the epidemiologists to make a study if they desire. We performed sensitivity tests by standard Kirby Bauer method using 4 hours broth culture. The choice of diagnostic media was dependent on the availability and the experience of the technician on its use. Whenever possible, multiple colonies of suspected non-lactose fermented colonies should be tested to look for *Shigella*, as on isolation plates colonies may differ culturally even in the same *Shigella* group.

In extreme conditions, the medium can be made by using a kerosene or gas stove and sterilized by kerosene or gas-heated autoclaves. A kerosene-heated refrigerator helps to prolong the shelf life of antiserum and the un-inoculated plates.

TABLE 1

COMPARATIVE ISOLATION OF DIFFERENT PATHOGENS IN DHAKA AND TEKNAF MICROBIOLOGY LABORATORY

	Total R/S Stool	Total Positive	*S. dysenteriae* type 1	*S. flex*	Other *Shigella*	*V. cholerae* 01	*V. cholerae* non 01	Salmonella
Dhaka Laboratory	15624	4460	182	748	189	2833	324	184
Teknaf Laboratory	8692	1312	16	1137	150	-	7	2

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Chapter 12

The Characterization of *Shigella* Toxins and Their Role in the Pathogenesis of Shigellosis

Gerald T. Keusch

Division of Geographic Medicine, Tufts University School of Medicine, Boston MA,

ABSTRACT

Shigellosis is a complex infection involving both small and large bowel, in which colonic epithelial cell invasion by the organism is a necessary event, and lumenal colonization of the small bowel may be critical as well. These events are poorly understood at present, especially in the human, and much work remains to be done.

The genus produces toxins which reproduce aspects of the disease in a variety of experimental models. While this permits a plausible explanation of pathogenesis, many aspects of the toxin action remain unknown. The hypothesis is testable, however, and further work can be planned and pursued now. When these data become available, it should be possible to develop and apply rational therapeutic and prophylactic interventions for this significant human disease.

Members of the genus *Shigella* cause an acute infection of the intestinal tract which is clinically manifested as a watery diarrhoea, often followed in a short time (hours to a day or two) by the dysentery syndrome (1). Virulence of the organism has been associated with its capacity to invade epithelial cells and to multiply within the intracellular compartment (2-5). The evidence to support this contention comes from many *in vitro* and *in vivo*

studies, including experimentally-induced infections in the natural primate hosts, humans and monkeys (4,5). The correlation between invasiveness and virulence can be demonstrated in cell culture systems (6), in the cornea of the guinea pig or rabbit (Sereney test) (4), and in experimental infections of guinea pigs and rhesus monkeys (2-4) and in human volunteers (5).

Fundamental to these studies has been the isolation of non-invasive variants of virulent strains, or the creation of *E.coli - Shigella* hybrids which initially invade but neither multiply nor survive within the cell, for these strains have proven to be incapable of causing disease (3). Non-invasive avirulent strains are readily obtained from *in vitro* cultures on nutrient agar by selecting opaque colonial transformants for study (4). While the majority of individual colonies derived from invasive, virulent isolates are translucent and retain their virulence attributes *in vivo*, a few opaque colonies appear which prove to be avirulent (7). A number of surface properties are altered in the opaque colonies including surface charge and detergent sensitivity (8,9), whereas the composition of the O-antigen repeat unit appears to be unchanged (10).

Although the mechanism by which such colonial variants are rendered non-invasive is unknown, the mechanism of the transformation has recently been suggested to be the loss of a large ∿ 120 Mdal plasmid (11). The strong correlation between colonial form *in vitro* and *in vivo* virulence is well established for a number of strains of *S.dysenteriae* and *flexneri*, and to a lesser extent, *S.sonnei* (2,4,5,6,12).

These data have been supported by experimental production of *E.coli - Shigella* hybrids. For example, hybrid *S.flexneri* 2a have been made which express *E.coli* O-antigens (13). Conversion to *E.coli* O-8 serotype, which shares a common immunodominant sugar (rhamnose) with *S.flexneri* 2a, fails to alter invasive properties or virulence. In contrast, insertion of genes expressing *E.coli* O-25 somatic antigen, in which the immunodominant sugar is mannose, results in simultaneous loss of invasiveness and virulence. Other hybrids containing portions of the *E.coli* genome invade intestinal cells but fail to establish intracellular colonization and are rapidly cleared within hours (3). These organisms are also clinically benign. The apparent requirement for intracellular multiplication is also supported by the finding that streptomycin-dependent mutants, unable to multiply in the absence of the antibiotic, are also avirulent (7). Indeed, such strains have been employed as experimental attenuated live oral vaccines with success (14).

Shigella Toxin(s):

The more controversial aspect of the pathogenesis of shigellosis concerns the involvement and role of cell-free protein toxins (1,4,5,10,15-17). The evidence for such a role at the present time is circumstantial, for it has thus far proven to be difficult to isolate a truly toxin-negative but invasive mutant to test *in vivo* (4,18). The problem in understanding toxins as virulence factors in shigellosis is complicated by the dual clinical presen-

tation of the disease as diarrhoea and dysentery (1), and the multiplicity of toxicities found in cell-free products derived from *Shigellae* (19). These problems must be considered first before the toxin(s) can be characterized and the evidence for their pathogenetic significance summarized.

Shigella Diarrhoea and Dysentery. Although shigellosis is classically described as dysentery, a clinical syndrome including abdominal cramps, painful daefecation (tenesmus), and passage of multiple stools per day (usually more than 20) characteristically of small volume and bloody-mucoid in appearance, the common initial presentation is that of a higher volume watery diarrhoea(1,5,12) (Table 1). The clinical presentation may be indistinguishable from that of toxigenic *E.coli* or rotavirus in young children. When patients are observed over time, the initial manifestation is generally diarrhoea, gradually giving way to the dysenteric form of the disease after a few hours or a few days *S.dysenteriae* 1 usually causes the most severe illness and the most rapid progression to dysentery, so rapid in fact that the phase of liquid stools can be overlooked (20). *S.sonnei* tends to be the most mild illness, often manifested as a self-limited diarrhoea without dysentery.

TABLE 1

CLINICAL - PATHOLOGICAL CORRELATIONS IN SHIGELLOSIS

Clinical Manifestation	Affected Site In Gut	Net Secretion of Fluid	Histologic Damage	Bacterial Invasion	Biochemical Mechanism
Watery Diarrhoea	Jejunum	Yes	No	No	?
Dysentery Syndrome	Colon	Yes	Yes	Yes	Inhibition of Protein Synthesis

Oral infection with *S.flexneri* 2a in the rhesus monkey has demonstrated a topological correlation of these two distinctive clinical pictures with anatomically distant portions of the intestinal tract, the proximal small intestine and the colon (21). In this model, watery diarrhoea is observed only in animals in which net jejunal secretion of isotonic fluid can be demonstrated, whereas dysentery occurs when infection causes an acute inflammatory colitis. When infection is induced by direct intracaecal introduction of the bacterial inoculum, watery diarrhoea is not observed and only dysentery results (22). In this case there is no alteration in transport of water and electrolytes in the small bowel and only the inflammatory lesions of the colon can be found.

In addition, whereas bacterial invasion of the inflammed colon is readily observed, there is no evidence of penetration of small bowel epithelium (21).

Rather only a lumenal population of bacteria is found at the site of jejunal secretion, a situation entirely comparable to the enterotoxic diarrhoeas such as cholera or toxigenic *E.coli*. In contrast, dysentery is correlated with an invasive bacterial colitis (4,5,10). These observations must be reconciled not only with the role of toxin in virulence, but also with the evidence presented above for the importance of invasion in pathogenesis.

Biological Activities of *Shigella* Toxin(s). The first description of cell-free toxins produced by the genus *Shigella* is generally attributed to Conradi in 1903 (23), although in the same year Neisser and Shiga (24) reported that intravenous administration to rabbits of filtered extracts of heat killed *S.dysenteriae* 1 was lethal in two days. The description by Conradi was more complete, however, and took note of the development of limb paralysis prior to death, which is a hallmark of the neurotoxin produced by the organism (Table 2). Employing autolysates of 18 hour cultures of *S. dysenteriae* 1, Conradi also showed that intraperitoneal injection into

TABLE 2

THE TOXINS OF THE GENUS *SHIGELLA*

	Neurotoxin	**Cytotoxin**	**Enterotoxin**
Route of administration	IV or IP	*in vitro*	topically to small bowel
Assay system	mice, rabbits	HeLa monolayers	ligated rabbit intestinal loops
Observed effects	limb paralysis followed by death	karyorrhexis and cell death	net fluid secretion accompanied by inflammation in ileum, but not jejunum
Antibody response	yes	yes	not reported
Relative toxicity	1	.001	.02

guinea pigs resulted in hypothermia and fatal collapse, indicative of the presence of lipopolysaccharide endotoxin, but no evidence of neurological involvement. Consistent with these observations, later studies have demonstrated that the guinea pig is at least 10,000-fold less sensitive to the neurotoxin on a per kg basis than the rabbit is (19). Within two years of this description, neurotoxin was demonstrated to be heat-labile, precipitated by ammonium sulfate, and antigenic, eliciting neutralizing antibody. Subse-

quent study has served to clearly distinguish this protein toxin from endotoxin, both chemically and biologically. Immunization of horses with endotoxin from different species of *Shigella*, produced species-specific agglutinating and precipitating antibodies, which protected mice against challenge by endotoxin and live bacteria, but not against neurotoxin. In contrast, immunization with neurotoxin produced from rough strains of *S. dysenteriae* 1 yielded antibody with high anti-neurotoxic activity in mice, but no agglutinating activity against the smooth variant (25).

Until recently, only *S. dysenteriae* 1 was reported to produce the neurotoxin, but this was due to suboptimal *in vitro* culture conditions and the lack of suitable methods to concentrate and isolate the toxin in other *Shigella* strains.

S. flexneri and *sonnei* have now been shown to produce less than 1/1000 of the amount of toxin obtained from *S. dysenteriae* 1 under identical conditions of growth (26,27). The dogma that the latter species alone of the *Shigellas* is toxigenic has been tumbled.

In 1960, a second biological property of *S. dysenteriae* 1 toxin was demonstrated by Vicari *et al* (28). Employing KB, monkey kidney, and human liver cells, they described a lethal cytotoxic action of the toxin on *in vitro* cultured monolayers (Table 2). Cytopathogenic effects preceded cell death; the cytotoxic effect was dose-dependent, was neutralized by anti-neurotoxic sera, and was 3700 times more sensitive than the neurotoxin action in mice. These results were confirmed by Mesrobeanu *et al* in 1962 (29), who described the toxin-induced, dose-dependent detachment of mammalian cells from the culture dish surface. Fifteen years later Keusch and Jacewicz and O'brien *et al* (26,27) extended these observations on the cytotoxic properties of cell free extracts of *Shigella* to *S. flexneri* and *sonnei*.

A third biological activity in toxin preparations from shigellae was first reported in 1970 by Keusch *et al* (Table 2). These workers isolated an enterotoxin from fractions of spent culture medium of *S. dysenteriae* 1, which induced isotonic fluid production in ligated segments of rabbit ileum (30). The same partially purified product was also neurotoxic in mice and cytotoxic to certain cells in culture (31). Cytotoxic effects to intestinal epithelial cells were also apparent in the rabbit ileum model exposed to toxin *in vivo*, preceding the accumulation of fluid within the ligated loops (32). By 6 hours after exposure to toxin detachment and death of intestinal epithelial cells occurred, creating microulcerations of the mucosa, with a marked inflammatory response within the lamina propria exuding into the bowel lumen. These studies thus experimentally reproduced the two cardinal features of clinical shigellosis with a cell-free product of the organism, secretion of isotonic fluid and an inflammatory enteritis of the gut mucosa, thus prompting the suggestion that a *Shigella* toxin was involved in disease pathogenesis (15,30).

Of further significance, when toxin was applied to the jejunum of the rabbit, a more conventional enterotoxin effect was observed, that is net secretion of isotonic fluid without histological abnormalities of the

mucosa (33). This is precisely the effect noted in jejunum during experimental infection of rhesus monkeys with *S. flexneri* 2a (21).

Production of Toxin(s). Both rough and smooth *Shigella* variants produce the three toxicities discussed above (19). A variety of media have been successfully employed for toxin production, including solid and liquid media. The latter require oxygenation (or agitation) for toxin to be elaborated, and none is obtained under anaerobic conditions (19). Synthesis of toxin is closely regulated by iron concentration of the medium. Below 0.1 µg Fe^{+++} per ml the organism grows poorly and toxin yields suffer, whereas when iron concentrations are raised above 0.15 ug per ml, toxin production itself progressively decreases. For example, yields decrease by over 60%, 90% and 98% when the iron content is increased to 0.2, 0.4 and 1.0 µg per ml, respectively (19).

During exponential phase growth, little toxin activity is present in culture media filtrates, but rather is extractable from the bacterial biomass itself (34). During the stationary phase of the growth cycle, toxin progressively appears in the medium as autolysis occurs (19). Toxin may be obtained from early logarithmic phase growth by French press lysis, the alkaline pH extraction method of van Heyningen, or by polymyxin B treatment, or from the medium after 24-to-48 hours of growth (19).

Characterization of Toxin(s). Activity of neurotoxin, cytotoxin and enterotoxin is heat-labile, but not exquisitely so. Bioactivity survives heating to 60°C for short periods of time; but is inactivated at 100°C for 30 minutes (19). Proteolytic enzymes also destroy toxicity (18), although mild trypsin or chymotrypsin treatment is reported to activate cytotoxin in a cell-free protein synthesis system derived from rabbit reticulocytes (35). Exposure of toxin to pH above 9.0 or below 5.5 sharply reduces cytotoxicity and neurotoxicity (19). Potentiation of enterotoxin activity in the ligated rabbit ileal loop by modest increases in pH above neutral has been reported (15).

Toxin Purification. It has been difficult to purify the neurotoxin of *Shigella* even though considerable bioactivity may be extracted from culture, because toxin represents only a tiny quantity of the proteins obtained (34). The purified neurotoxin obtained by van Heyningen and Gladstone in 1953 has, on subsequent examination by SDS - polyacrylamide gel electrophoresis, proven to be contaminated by many non-toxin components (19). Great progress has been made in the past few years employing modern techniques of protein chemistry. These studies have utilized the cytotoxin action in cell culture, to follow the toxin during purification through several steps, including molecular sieve chromatography, blue sepharose column chromatography, ion exchange chromatography (both cationic and anionic resins), affinity chromatography on antibody or chitin columns, and polyacrylamide gel electrophoresis with and without SDS. The results are similar, though not identical, in several laboratories employing somewhat distinctive steps in the purification scheme (19,35-38). Purified cytotoxin contains a major polypeptide of 30,000-33,000 dal, which may be composed of a large 27,000-29,000 dal fragment and a small 3-4,000 dal fragment. In addition, a second major peptide with a molecular weight variously estimated at 5,000-11,000 dal is also present.

Crosslinking studies by Olsnes *et al* (37), the most comprehensive studies to date, indicate that the toxin is composed of an A subunit of 30,000 dal, made of an A1 fragment (27,000 dal) and an A2 fragment (3,000 dal), and a B subunit, composed of 6-8 monomeric units of about 5,000 dal. The molecular weight of the native cytotoxin is estimated to be around 64,000 - 72,000 dal. The purified cytotoxin appears to possess both enterotoxin and neurotoxin activities as well, but it is still uncertain that a single molecule is responsible.

Mechanism of Action. A clear understanding of the cytotoxic action in cell culture systems is now possible. The first step is the binding of the toxin to the cell membrane (39). In the HeLa cell, the membrane receptor has been partially characterized as a lysozyme-sensitive glycoprotein with $\beta 1 \rightarrow 4$ linked N-acetyl-D-glucosamine oligomers involved in receptor specificity. Following binding, toxin is rapidly removed from the cell surface by an energy-dependent process, inhibitable by a variety of metabolic poisons and cold temperature (40). Toxin transport has the biochemical characteristics of receptor-mediated endocytosis, as cytotoxicity is prevented by amines (NH_4Cl_2, methylamine, dansylcadaverine, putrescine, etc) that inhibit other well-characterized receptor-mediated endocytic systems, and by agents that inhibit the cytoskeleton involved in internalization of endocytic vesicles and fusion with lysosomes (cytochalasin-B, colchicine) (40). However, morphological confirmation of toxin uptake has not yet been demonstrated. Following internalization, the toxin must escape the vesicle in a biologically active form, for its target is within the cytoplasm (17). There is, most likely, a processing step within the vesicle, for drugs that concentrate in and inhibit lysosomal function (chloroquine, corticosteroids) also protect the toxin-treated cell (40). The earliest detected biochemical effect of toxin is the inhibition of cellular protein synthesis (36,41). Cell-free ribosomal protein-synthesizing systems from HeLa cells, rabbit reticulocytes or a wheat-germ-globin m-RNA system are also inhibited by toxin *in vitro* (35,37,42). Olsnes and colleagues have recently reported that the target of the toxin action is the 60S ribosomal subunit, and that an irreversible catalytic event occurs which prevents peptide chain elongation (42).

Infection of HeLa cells with toxin-producing virulent organisms also inhibits protein synthesis, and there is evidence to correlate this effect with the intracellular production of toxin (6). Invasion of the same cell by a non-toxigenic *Salmonella* strain neither affects protein synthesis nor cell viability.

Based on the *in vivo* effects of toxin on the central nervous system or in the ligated rabbit ileum, it is reasonable to suggest that a similar cytotoxic action on vascular endothelium in the CNS or on the ileal epithelial cell can explain the neurotoxin action and the inflammatory component of the enterotoxin activity. Biochemical data to support this concept have not been reported as yet.

The mechanism of the secretory enterotoxin effect is uncertain. Although resembling the action of cholera toxin or *E.coli* LT in the rabbit jejunum (33),

there is conflicting evidence regarding the ability of *Shigella* toxin to activate adenylate cyclase and to increase the intraepithelial cell level of cyclic AMP (43,44). Elevation in rabbit intestinal mucosal c-AMP following *in vivo* exposure to *Shigella* toxin has been reported, However, the increase occurred after, and not before, fluid secretion began and, therefore, may be the result, and not the cause, of altered gut function (45). Conflicting reports have also appeared with respect to the cell culture systems responsive to cholera and *E.coli* LT toxins, the Y-1 adrenal cell and the CHO cell. Whereas the former did not respond to *Shigella* enterotoxin preparations (46), Takeda has reported the finding of a CHO cell toxin in various species of *Shigella* that is distinct from the neurotoxin and cytotoxin (47). Effects of the CHO cell toxin in the rabbit ileum were not reported, however.

Ketyi and coworkers reported finding a heat-stable enterotoxin in *Shigella flexneri*, which was cytotoxic in cell culture and enterotoxic in the suckling mouse model for *E.coli* ST (48). *E.coli* ST appears to activate jejunal guanylate cyclase and raise the intracellular level of c-GMP (49). In turn, this decreases the absorptive flux of isotonic fluid in the jejunum, eventuating in net secretion in the bowel. No direct evidence has been presented that the material isolated by Ketyi is similar in its action, and, indeed, it differs dramatically from *E.coli* ST in reported physical properties, and is only questionably active in rabbit loops.

Shigellosis as a Toxin-Mediated Disease. While it is known that various *Shigella* species produce toxin(s) *in vitro*, it is important to ask at the outset if there is any evidence of toxin production *in vivo* during human infection? Toxin itself has not yet been detected in intestinal fluids or in stool, However, serum antibody to the cytotoxin has been demonstrated in patients with *S.dysenteriae*, *flexneri* and *sonnei* infections (18,26,50). In serial samples from patients with naturally acquired *S.dysenteriae* type 1 disease during the 1969 Central American epidemic, antibody was present within the first week of symptomatic disease, rapidly reached a plateau titer, and disappeared sometime after 9 months post-infection (50). Studies of volunteers experimentally infected with the epidemic strain or a hypotoxigenic laboratory mutant confirmed the rapid rise in titer in the wild type strain, a somewhat delayed response in the mutant strain, and a decrease in titer after 50 days of observation (18). Fractionation of the antibody by sucrose density ultracentrifugation and by specific antibody-affinity chromatography showed that the antibody response was of the IgM type. Subsequent studies in patients infected with *S.flexneri* or *S.sonnei* confirmed the general characteristics of the antibody response to the toxin, and clearly indicated the production of toxin antigen during disease (26). These data remain the only evidence of toxin production *in vivo*.

The rest of the argument for the role of toxin is, as noted earlier, circumstantial. The toxin can cause the cardinal manifestations of shigellosis *in vivo* in experimental animals (1,15,32). The secretory response with no histologic derangement induced by toxin in proximal rabbit small bowel (33) is consistent with the jejunal response to live *S.flexneri* 2a infection in rhesus monkeys, in which neither bacterial invasion nor mucosal

inflammation occur in the colonized, secreting segment of bowel (21). Although DuPont and Pickering (51) have found evidence for a lumenal population of organisms during induced *S.flexneri* 2a infections of humans, Levine *et al* could not confirm the observation in volunteers infected with *S.dysenteriae* type 1 (5). Further studies will be required to explain these discrepancies, and to demonstrate whether or not shigella enterotoxin is present in the lumen at the site of fluid secretion.

The colonic phase of shigellosis is quite distinct – an invasive inflammatory colitis is the hallmark of dysentery (1,4,5,10,16,21,22). The available data indicate that colonic epithelium is not affected by topically administered toxin (52,53). Yet the histologic effects of the invasive bacterial infection are reproduced by cell-free soluble toxin in the rabbit ileum (32), which thus appears to possess the toxin receptors apparently missing or perhaps functionally masked in colonic tissue. It has been suggested that bacterial invasion of colon serves to overcome the lack of cell surface toxin receptors, by producing toxin intracellularly, close to the ribosomal target of the cytotoxin as defined in the HeLa cell model (1,15,16,17,19,40). This concept has been supported recently by the finding that tissue culture cells refractory to exogenously administered *Shigella* toxin nonetheless demonstrate inhibition of protein synthesis and concomitant cytotoxicity, when invaded by toxigenic *Shigellas* but not by invasive, non-toxigenic *Salmonellas* (6). These data are thus consistent with the role of toxin in pathogenesis of colitis and dysentery, but do not constitute proof of the suggested mechanism. It should be obvious that adequate evidence will be difficult to obtain. Preimmunization of the host, even if successful in inducing secretory antibodies at the gut surface, will have little or no role to play in the intracellular milieu wherein the postulated events take place. Direct evidence will thus require demonstration of toxin within infected colonic cells (a technically difficult task at present if a few hundred toxin molecules are all that is required to totally inhibit protein synthesis and to kill the cell), or, at minimum, a biochemical demonstration of the specific effect of the toxin on the 60S ribosomal subunit, whatever that may be (42). Development of a truly non-toxinogenic but still invasive mutant, deleted for structural genes of the active subunits of the toxin, would also constitute reasonable proof of the hypothesis, if this organism were to prove to be incapable of initiating colitis and dysentery *in vivo*. Thus far, however, only hypotoxinogenic mutants have been found, and these clearly remain virulent and induce a serum antitoxin response (4,5,18).

The Therapeutic Challenge of the Toxin Hypothesis. The concept that watery diarrhoea in shigellosis is due to local, lumenal toxin production by a colonizing population of bacteria like cholera or toxigenic *E.coli* presents the challenge to interrupt colonization, to prevent toxin-epithelial cell interactions, or to pharmacologically prevent or reverse the induced fluid response in the gut (17). We know very little at present about any of these approaches in shigellosis. Potentially, they could be exceedingly effective, especially in *S.sonnei* disease, which often does not progress beyond the watery diarrhoea phase.

The unique challenge of shigellosis is the dysentery syndrome, so common in both *S. dysenteriae* 1 and *S. flexneri* infection. Here the approach must concentrate on the invasive process itself, whereby the organism penetrates the epithelial cell, the conditions under which intracellular bacterial multiplication and toxin production occur, and development of specific intracellularly acting drugs, which prevent or reverse the biochemical lesion caused by the toxin. It is safe to say that we know nothing about these events at the present time.

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Chapter 13

Genetic Studies on the Virulence of Dysentery Bacilli

Samuel B. Formal, Philippe Sansonetti and Dennis J. Kopecko

Walter Reed Army Institute of Research, Washington, D.C.

The virulence of dysentery bacilli is associated with their ability to invade (1) and multiply in the epithelial cells of the colonic mucosa (2). Although man and sub-human primates are the only natural hosts for *Shigellae*, laboratory assays are available which are reasonably reliable indicators of a dysentery organism's ability to invade epithelial cells. The assays which our laboratory employs include determining whether or not an isolate will invade HeLa cells, produce a positive Sereny test, kill starved, opiated guinea pigs, or invade and produce fluid in a rabbit ileal loop.

We and others have used these models together with microbial genetic techniques to study the biologic properties of *Shigellae*. Several chromosomal loci appear to be required for virulence. If the *PurE* region of the chromosome of virulent *S. flexneri* is replaced by that of *E. coli* K-12, the resulting *S. flexneri* hybrids are uniformly unable to produce keratoconjunctivitis (3). Conversely, avirulent colonical mutants of *S. flexneri* have been repaired in one case by transferring the *gl* K region from a virulent donor (4), and in another case the *mal* B region from an *E. coli* K-12 donor strain (5). Other studies have identified the *his* chromosomal region with the expression of the group antigen of *S. flexneri* and the *pro* region with the synthesis of the type-specific antigen (6).

More recently, interest has been focused on the role of plasmids in the biological properties of *Shigellae*. Initial studies were carried out with virulent *S. sonnei*. These organisms, termed form I, dissociate at a high frequency, to yield rough avirulent variants, termed form II. The form I to form II variation is irreversible and is accompanied by the loss of the form I antigen. Accordingly, the plasmid profiles of various *S. sonnei* strains,

isolated from patients in different parts of the world, were examined. All form I strains contained a large plasmid of approximately 120 Mdal in size; this plasmid is absent in avirulent form II derivatives (7). A similar observation has been made independently by Sansonetti et al (8).

Direct proof that this large plasmid is involved in form I antigen synthesis and virulence can only be obtained by reintroduction of this plasmid into a form II recipient cell, with concomitant reestablishment of these properties. However, neither the form I antigen nor virulence phenotypes are useful as selective markers to monitor plasmid transfer. Therefore, we attempted to identify any marker of selective value expressed by the form I plasmid. To date, about 175 biochemical and antibiotic-resistance characters have been tested for, but we have been unable to detect any other trait encoded on this large plasmid. To circumvent this problem, the form I plasmid was phenotypically tagged with the ampicillin resistance transposon, Tn*3*, or with transposons Tn*5* and Tn*10*. These transposons were introduced into the appropriate stains on a carrier F'$_{ts}$ *lac* replicon that is temperature sensitive for replication. Strains in which the form I plasmid had been tagged expressed the appropriate transposon-encoded antibiotic resistance; and, this resistance was always lost during the transition to form II cells.

Attempts to detect conjugal self-transfer of these tagged plasmids, using antibiotic selective pressure, were unsuccessful, indicating that these large plasmids are not self-transmissible. However, two systems to mobilize the form I plasmid to recipient cells have been developed. Initially, an F'$_{ts}$ *lac*: Tn*3* plasmid was introduced into a *S. sonnei* strain carrying a Tn*3*-tagged form I plasmid. We reasoned that recombination between the Tn*3* units on these two plasmids would result in the formation of a composite conjugative plasmid. In fact, form I plasmid transfer was obtained, as well as evidence for the composite plasmid species. Using this mobilization system, form I antigen synthesizing ability has been transferred to form II *S. sonnei*, *S. flexneri*, *E. coli* K-12, and *Salmonella typhi* (7). A second procedure used to transfer the form I plasmid to recipient form II strains involves mobilization of the Tn*5* transposon-tagged form I plasmid by the plasmid R386. Such conjugal matings also resulted in restoration of the ability of form II strains to express the form I antigen (9). These data strongly suggest that this *S. sonnei* plasmid carries the structural genes for the form I antigen.

Plasmids also play a role in the pathogenic properties of *S. flexneri*. Representative strains of the six serotypes of *S. flexneri* have been examined for plasmids. Regardless of serotype, all strains were found to contain multiple plasmid species and always contained at least one large plasmid species of approximately 140 Mdal in size (unpublished data). Virulent, smooth *S. flexneri* colonies, when restreaked and grown at 42°C, lose this large plasmid at a frequency of approximately 1 percent. Strains in which the large plasmid has been eliminated are still smooth in colonial morphology but are always avirulent and unable to penetrate epithelial cells. Recently, this large *S. flexneri* plasmid in one serotype has been tagged with the Tn*5* transposon. Although this tagged plasmid is not self-transmissible, it has been mobilized by one of several conjugative drug resistance plasmids. When avirulent *S. flexneri* strains lacking this large plasmid receive the Tn*5*-

tagged *S. flexneri* plasmid via mobilization, these transconjugants regain virulence (10, manuscript in preparation). These data strongly demonstrate that plasmid-borne genes are involved in conferring invasive ability to *S. flexneri*. However, the exact plasmid-mediated virulence functions involved are still undetermined.

Thus, the combined use of laboratory models for virulence and microbial genetics have been a valuable approach to the study of the biological properties of dysentery bacilli and in defining some of the stages in the pathogenesis of the disease. In addition, these procedures have also been useful in constructing living attenuated oral vaccines and for devising tests to develop the safety of these products. It is most likely that as the newer techniques of molecular biology become available to laboratories working on problems of pathogenesis, our knowledge of disease processes will be greatly increased, and improved methods for preventing and treating diarrheal diseases will be developed.

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Chapter 14

Toxins from *Shigella*

Yoshifumi Takeda, Keinosuke Okamoto[1], Tomomasa Yano[2], Takeshi Honda and Toshio Miwatani

Department of Bacteriology and Serology, Research Institute for Microbial Diseases, Osaka University, Japan.[1] Present address: Department of Microbiology, School of Medicine, Fujitagakuen University, Toyoake, Japan. [2] Present address: Department of Microbiology and Immunology, University of Campinas, Sao Paulo, Brazil.

ABSTRACT

Only 5 years after the discovery of *Shigella dysenteriae* by Shiga (1898), Conradi (1903) reported that the organism produces a toxin that causes paralysis followed by death. Later, Olitsky and Klinger (1920) demonstrated that toxic preparations contained at least two biological active materials: a protein antigenic toxin named neurotoxin that was responsible for the neurological symptoms and lipopolysaccharide endotoxin. Although numerous works on the neurotoxin have since been reported (Engley, 1952; van Heyningen, 1971), the toxin has not yet been purified sufficiently to study its physicochemical and biological properties. Engley (1949) reported some physicochemical properties of a purified neurotoxin, but he did not demonstrate the homogeneity of the material. Van Heyningen and Gladsone (1953) also reported extensive studies on the purification of the neurotoxin extracted from cells of *S.dysenteriae*, but this material was later used by Keusch *et al* (1976), and found to give multiple protein bands on polyacrylamide gel electrophoresis. Sourek and Raska (1963) reported purification of the neurotoxin by preparative electrophoresis and Sephadex G-50 gel filtration, but did not give detailed information on the purification and homogeneity of the purified material. Large scale purification of the neurotoxin from the culture filtrate of *S.dysenteriae* was described by McIver *et al* (1975). They obtained highly purified neurotoxin, which showed both cytotoxicity to HeLa cells and enterotoxicity in the rabbit ileal loop test, by a single run on iso-

electrofocusing electrophoresis on polyacrylamide gel. However, further analysis showed that the material contained two toxic fractions: one showing neurotoxicity, cytotoxicity and enterotoxicity, and the other only cytotoxicity. Thompson *et al* (1976) developed a purification procedure which included ammonium sulfate precipitation DEAE-Sephadex chromatography, Biogel A-0.5m gel filtration, isoelectrofocusing and polyacrylamide gel electrophoresis. More recently, purification of the neurotoxin by affinity column chromatography using BrCN-activated Sepharose 4B coupled with antineurotoxin antiserum, has also been reported (O'Brien *et al* 1980).

This paper reports our results on purification of the neurotoxin from the culture supernatant of *S.dysenteriae* 1. The purification procedure involves ammonium sulfate fractionation, column chromatographies on DEAE-cellulose, CM-cellulose, hydroxylapatite, and gel filtration on Sephadex G-200. Neurotoxic activity was assayed by measuring its lethal effect on intraperitoneal injection into mice, and about 4,760-fold purification was achieved with a yield of 2.7%. The purified neurotoxin appeared homogeneous on polyacrylamide gel disc electrophoresis and consisted of two subunits. The 50% lethal dose (LD_{50}) of the purified neurotoxin was 0.15 mcg/mouse. Intraperitoneal injection of 2 LD_{50} of the purified neurotoxin into mice caused paralysis of the limbs followed by death after 2 to 4 days.

Some other biological and pathological properties of the purified neurotoxin will be discussed.

INTRODUCTION

Production of a lethal toxin by *Shigella dysenteriae* was first reported by Conradi (1) only 5 years after the discovery of this organism by Shiga (2,3). Olitsky and Kliger (4) showed that toxic preparations from *S.dysenteriae* contained at least two biologically active materials: a protein-antigenic toxin that was responsible for the neurological symptoms, which they name "neurotoxin", and a lipopolysaccharide endotoxin. The protein toxin (neurotoxin) has since been studied extensively by several workers. Engley (5,6) reported some physicochemical properties of a purified neurotoxin, but he did not demonstrate the homogeneity of his material. Van Heyningen and Gladstone (7) also purified a neurotoxin from cells of *S. dysenteriae*, but this material was later found by Keusch *et al* (8) to give multiple protein bands on polyacrylamide gel electrophoresis. Šourek and Raške (9) reported purification of the neurotoxin by preparative electrophoresis and Sephadex G-50 gel filtration. Large-scale purification of the neurotoxin from the culture filtrate of *S.dysenteriae* was described by McIver *et al* (10). They obtained highly purified neurotoxin, which showed both cytotoxicity to HeLa cells and enterotoxicity in the rabbit ileal loop test, by a single run on isoelectrofocusing electrophoresis on polyacrylamide gel. However, further analysis showed that the material contained two toxic fractions: one showing neurotoxicity, cytotoxicity and enterotoxicity,

and the other only cytotoxicity. Thompson *et al* (11) developed a purification procedure that included ammonium sulfate precipitation, DEAE-Sephadex chromatography, Bio-Gel A-0.5 m gel filtration, isoelectrofocusing and polyacrylamide gel electrophoresis. More recently, O'Brien *et al* (12) purified the neurotoxin by affinity column chromatography on BrCN-activated Sepharose 4B coupled with anti-neurotoxin antiserum. Olsnes and Eiklind (13) also reported purification of a toxin, which they designated as cytotoxin, by adsorption to, and elution from, a column of acid-treated chitin, followed by DEAE-cellulose column chromatography and sucrose density-gradient centrifugation. This paper describes a purification procedure recently developed in our laboratory (14, 15) and some properties of the purified neurotoxin.

It has been reported that the purified neurotoxin shows enterotoxicity when tested for its effect in causing fluid accumulation in rabbit ileal loops (10, 16). However, the mechanism of fluid accumulation by the neurotoxin of *S.dysenteriae* might be different from that of cholera enterotoxin (17), which stimulates intestinal mucosal adenylate cyclase and, thus, increases the intracellular concentration of cyclic AMP. Although Charney *et al* (18) reported activation of intestinal mucosal adenylate cyclase by *Shigella* toxin, there is no convincing evidence that the mechanism of action of the neurotoxin is mediated by cyclic AMP. We tried to isolate cholera-like enterotoxin from culture supernatants of *Shigella*, and demonstrated the existence of a factor that causes morphological changes of Chinese hamster ovary (CHO) cells (19, 20). The distribution, purification and possible enterotoxicity of this factor are described in this paper.

PURIFICATION OF THE NEUROTOXIN

S.dysenteriae 1 (RIMD 3101010), provided by Dr. M.I. Huq, International Centre for Diarrhoeal Disease Research, Bangladesh, was cultured in medium consisting of 10 g of peptone (Difco), 5 g of yeast extract (Difco), 10 g of Na_2HPO_4 and 5 g of glucose per litre in distilled water at 37°C for 24 hours with vigorous shaking. The supernatant was obtained by centrifugation of the culture at 15,000 x g for 20 minutes. Solid ammonium sulfate was added to the culture supernatant (56 g/100 ml) at 4°C, and the resulting precipitate was dissolved in a small volume of 0.01 M phosphate buffer (Na_2HPO_4 - KH_2PO_4, pH 7.0) and dialyzed overnight against the same buffer. The dialyzed sample was used as crude toxin after removal of insoluble material by centrifugation at 20,000 x g for 20 minutes.

The crude toxin was purified by successive column chromatographies on DEAE-cellulose, CM-cellulose, hydroxylapatite and Sephadex G-200. The toxin was assayed by injecting 0.5 ml of samples intraperitoneally into mice 4 - 5 weeks old. Animals were observed for 7 days, and death after 2 - 7 days were considered to be due to the neurotoxin. The neurotoxin was not adsorbed on a DEAE-cellulose column and, thus, was eluted with 0.01 M

phosphate buffer (pH 7.0). The neurotoxin was adsorbed to CM-cellulose column and eluted with 0.2 M NaCl in 0.01 M phosphate buffer. The neurotoxin eluted from the CM-cellulose column was applied to a hydroxylapatite column and material was eluted stepwise with 0.01 M, 0.05 M, 0.1 M and 0.2 M phosphate buffer (pH 7.0). The neurotoxin was eluted with 0.1 M Phosphate buffer (pH 7.0), and chromatographed further on Sephadex G-200 gel.

The LD_{50} values of samples at step of purification were measured. The LD_{50} values of the culture supernatant, crude toxin DEAE-cellulose column eluate, CM-cellulose column eluate, hydroxylapatite column eluate and Sephadex G-200 column eluate were 714 µg, 115 µg, 85 µg, 3.2 µg, 0.7 µg and 0.15 µg per mouse, respectively (Table 1). Typical values for purification of the neurotoxin are summarized in Table 2. The neurotoxin was purified about 4,760-fold from the culture supernatant, with a recovery of about 2.7%.

The homogeneity of the purified neurotoxin was examined by polyacrylamide gel disc electrophoresis. As shown in Figure 1, the purified neurotoxin gave a single stained band on the gel. To demonstrate that the stained band corresponded to the neurotoxin, the polyacrylamide gel was cut into sections of 2 mm width after electrophoresis, and these were suspended in phosphate-buffered saline (pH 7.0). Assay of the extracts for their lethal effects on mice showed that the neurotoxin migrated in the same position as the stained band. These data show that the neurotoxin was purified almost to homogeneity.

MOLECULAR STRUCTURE OF THE PURIFIED NEUROTOXIN

The molecular weight of the purified neurotoxin was determined by Sephadex G-100 gel filtration. As shown in Figure 2, the molecular weight of the purified neurotoxin as calculated to be about 42,000.

On SDS-polyacrylamide gel disc electrophoresis, the purified neurotoxin migrated as two stained bands (Figure 3), indicating that it consisted of two subunits with different molecular weights. Use of marker proteins showed that the molecular weights of these subunits were about 35,000 and 10,000 (Figure 4). Since the molecular weight of the neurotoxin determined by Sephadex G-100 gel filtration was about 42,000 (Figure 2), it is concluded that the neurotoxin consists of 1 molecule each of these two subunits.

O'Brien *et al* (12) and Olsnes and Eiklid (13) also reported that the neurotoxin has a subunit structure. The data of Olsnes and Eiklid (13) are most similar to ours, but they suggested that the native toxin consists of one heavy subunit and 5 light subunits.

TABLE 1

LETHAL EFFECTS OF FRACTIONS AT VARIOUS STEPS OF PURIFICATION

Fraction	Amount of protein (μg) injected/mouse	No. of mice that died/no. of mice examined
Culture supernatant	4,400	10/10
	2,200	8/10
	1,100	6/10
	220	2/10
	44	0/10
Crude toxin	1,100	10/10
	220	7/10
	44	2/10
	8.8	0/10
DEAE-cellulose column eluate	950.0	10/10
	190.0	7/10
	38.0	3/10
	7.8	0/10
CM-cellulose column eluate	35.0	10/10
	7.0	7/10
	1.4	3/10
	0.3	0/10
Hydroxylapatite column eluate	6.5	10/10
	1.3	6/10
	0.26	3/10
	0.05	0/10
Sephadex G-200 column eluate	1.0	10/10
	0.33	7/10
	0.10	4/10
	0.01	0/10

TABLE 2

PURIFICATION OF NEUROTOXIN FROM CULTURE SUPERNATANT OF *S.DYSENTERIAE*

Fraction	Total vol. (ml)	Total protein (mg)	Total activity (LD_{50})	Specific activity (LD_{50}/mg)	Relative activity	Yield of lethal activity %
Culture supernatant	46,800	351,000	501,429	1.4	1.0	100.0
Crude toxin	1,170	35,098	305,217	8.7	6.2	60.9
Deae-cellulose column eluate	900	21,330	250,941	11.8	8.4	50.0
CM-cellulose column eluate	245	129.6	40,500	312.5	223.2	8.1
Hydroxylapatite column eluate	18.0	20.4	29,143	1,428.6	1,020.4	5.8
Sephadex G-200 column eluate	2.2	2.04	13,600	6,666.7	4,761.9	2.7

Fig. 1: Polyacrylamide gel disc electrophoresis of the purified neurotoxin.

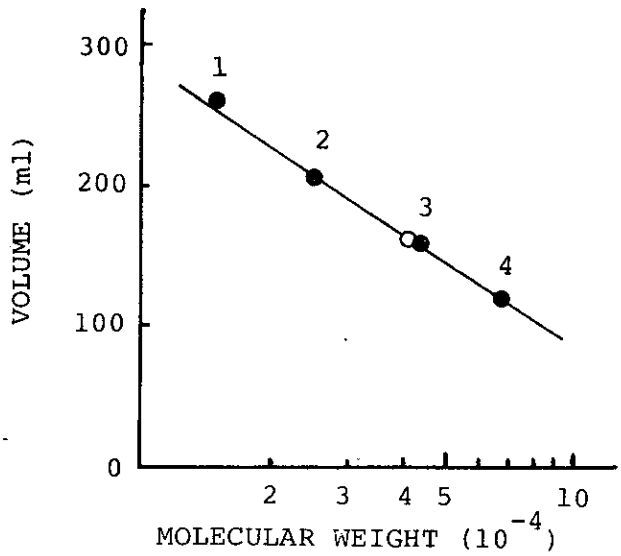

Fig. 2: Determination of molecular weight of the purified neurotoxin by Sephadex G-100 gel filtration. O, Purified neurotoxin; ●, standard proteins: 1, cytochrome c; 2, chymotrypsinogen A; 3, ovalbumin; 4, bovine serum albumin.

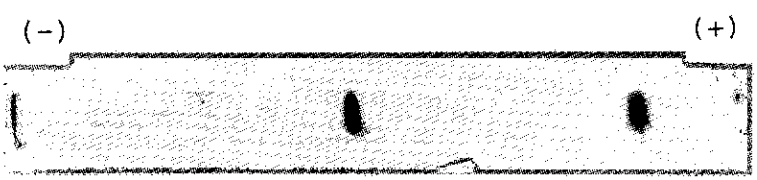

Fig. 3: SDS-polyacrylamide gel disc electrophoresis of the purified neurotoxin.

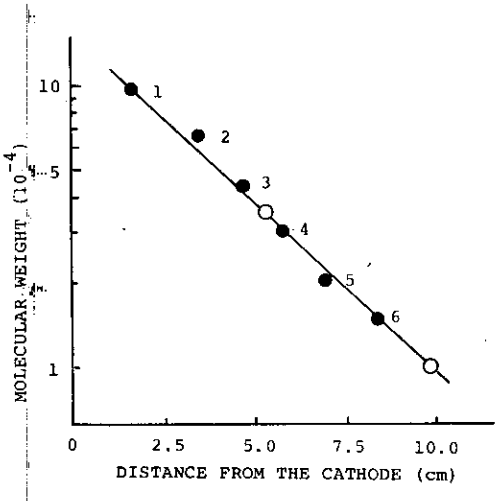

Fig. 4: Determination of molecular weight of the purified neurotoxin by SDS-polyacrylamide gel disc electrophoresis. O, Purified neurotoxin; ●, standard proteins: 1 phosphorylase b; 2, bovine serum albumin; 3, ovalbumin; 4, carbonic anhydrase; 5, soybean trypsin inhibitor; 6, α-lactalbumin.

ISOLATION AND PURIFICATION OF A FACTOR FROM THE CULTURE FILTRATE OF *SHIGELLA* THAT CAUSES MORPHOLOGICAL CHANGE OF CHO CELLS

A toxic factor that causes morphological change of CHO cells (CHO-factor) was isolated and purified from *S. dysenteriae* 1 (RIMD 3101010) (19, 21) and from *S. sonnei* (RIMD 3104007) (14). The crude toxin prepared for purification of the neurotoxin, which was obtained by concentrating the culture filtrate of *S. dysenteriae* by ammonium sulfate precipitation as described in the preceding section, was chromatographed on a DEAE-cellulose column equilibrated with 0.01 M phosphate buffer (pH 7.0). The column was eluted successively with 0.01 M phosphate buffer (pH 7.0) containing 0.2 M and 0.9 M NaCl. The two fractions obtained were each concentrated by adding solid ammonium sulfate (56 g/100 ml), dialyzed against 0.01 M phosphate buffer (pH 7.0), and designated as fractions I and II.

As shown in Table 3, lethal toxicity to mice on intraperitoneal administration was eluted in faction I, but not in fraction II. On the other hand, the activity causing morphological changes of CHO cells was found almost entirely in fraction II. These results suggest that the CHO-factor is distinct from the neurotoxin. Fraction II was applied to a Bio-Gel A-5m column, and the CHO-factor eluted with 0.01 M phosphate buffer (pH 7.0) was concentrated by vacuum dialysis against 0.001 M phosphate buffer (pH 7.0) The concentrate was then applied to a hydroxylapatite column equilibrated with 0.001 M phosphate buffer (pH 7.0). Material was eluted successively with 0.001 M, 0.01 M and 0.2 M phosphate buffer (pH 7.0); the CHO-factor was eluted with 0.01 M phosphate buffer (pH 7.0). The CHO-factor thus purified caused morphological changes of CHO cells, although its activity was about 1/1,000 that of the purified cholera enterotoxin (Figure 5). Typical pictures of CHO cells treated with the CHO-factor from *S. dysenteriae* are shown in Figure 6.

The CHO factor was further purified by successive column chromatographies on DEAE-Sephadex A-20 and Sephadex G-100. The final preparation gave only one band on polyacrylamide gel disc electrophoresis (Figure 7). The mobility of the purified preparation on polyacrylamide gel disc electrophoresis differed from that of the purified neurotoxin shown in Figure 1, supporting the idea that the neurotoxin and CHO-factor are distinct molecules.

TABLE 3

MOUSE LETHAL ACTIVITY OF FRACTIONS ELUTED FROM A DEAE-CELLULOSE COLUMN

Fraction	Amt of protein (mg) injected/mouse	No. of mice that died/no. of mice examined
I	12.0	10/10
	3.0	4/10
	0.3	0/10
II	12.0	0/10
	3.0	0/10
	0.3	0/10

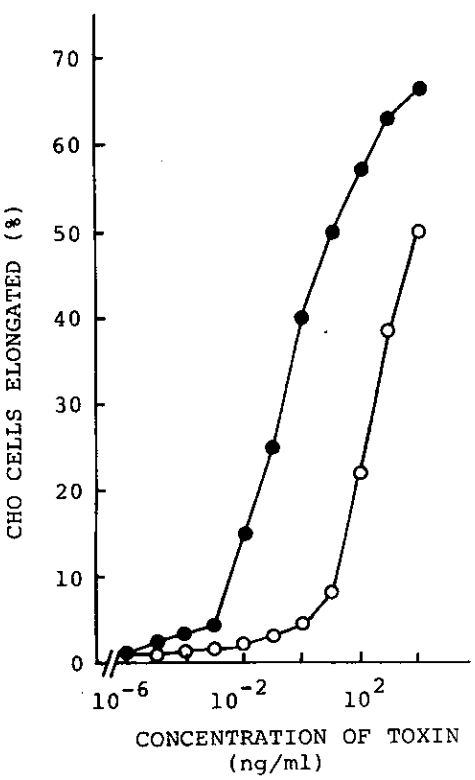

Fig. 5: Effect of partially purified CHO-factor from *S.dysenteriae* cholera enterotoxin on the morphology of CHO cells. ○, CHO-factor from *S.dysenteriae;* ●, cholera enterotoxin.

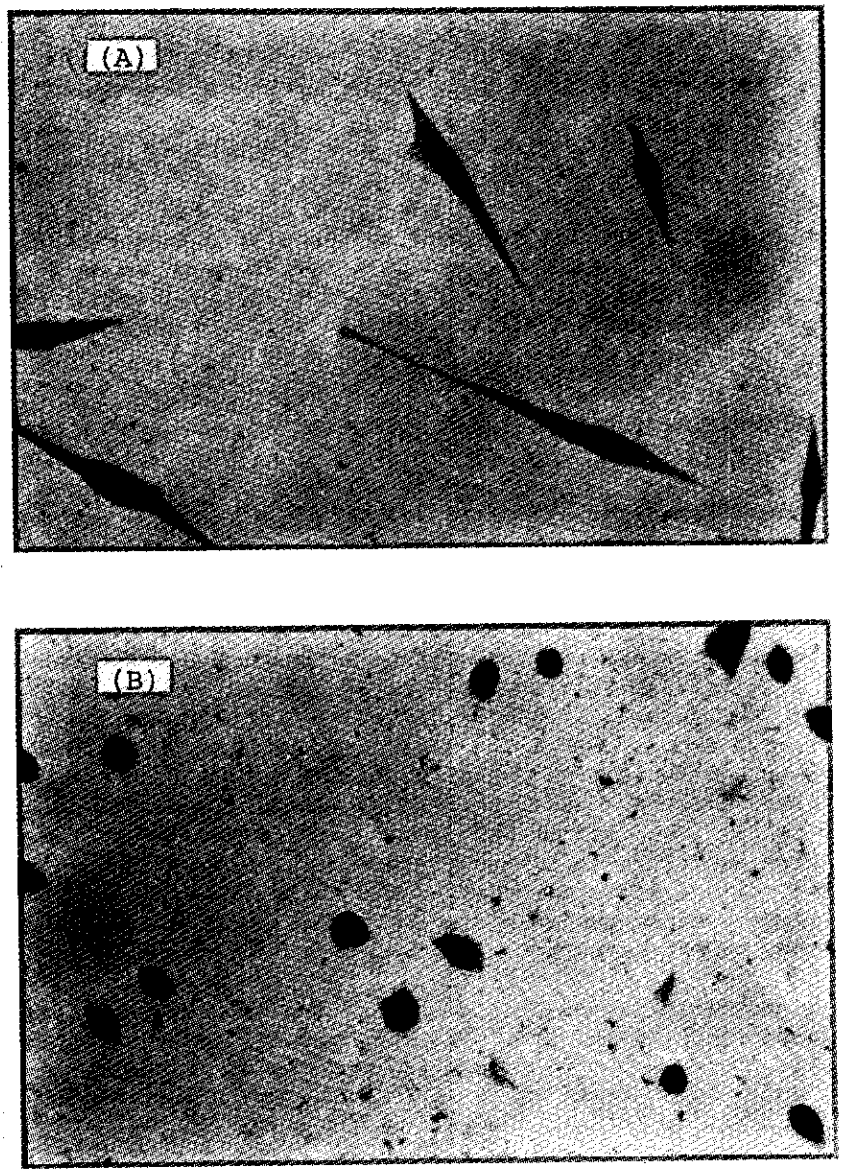

Fig. 6: Morphological changes of CHO cells caused by partially purified CHO-factor from *S. dysenteriae*. (A) Spindle-shape of cells induced by the CHO-factor. (B) Oral control cells.

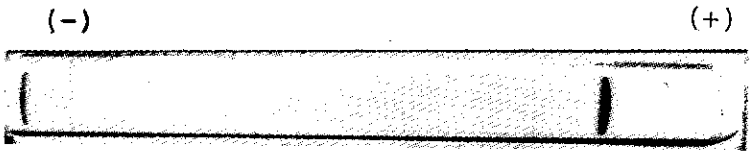

Fig. 7: Polyacrylamide gel disc electrophoresis of purified CHO-factor.

The purified CHO-factor gave positive vascular permeability reactions on rabbits' skin. However, it has not yet been demonstrated to be an enterotoxin by the rabbit ileal loop test. Thus, further study is necessary to characterize the CHO-factor and to elucidate its role in *Shigella* infection.

DISTRIBUTION OF CHO-FACTOR IN *SHIGELLA* SPECIES

The distribution of the CHO-factor in *Shigella* species was determined by examining culture filtrates of various strains of *Shigella*. Thirty-nine strains, namely 17 strains of *S.dysenteriae*, 15 strains of *S.flexneri*, 4 strains of *S.boydii*, and 3 strains of *S.sonnei*, were cultured in brain heart infusion broth (Difco) at 37°C for 24 hours with vigorous shaking. The culture supernatants were obtained by centrifugation at 20,000 x g for 30 minutes and their ability to cause morphological changes of CHO cells was examined. The results are shown in Table 4. The percentage elongation of CHO cells ranged from 1.3 to 12.5%, depending upon the *Shigella* strain tested. The culture supernatants of *S.dysenteriae* RIMD 3101005 and RIMD 3101003 and *S.flexneri* RIMD 3102015 caused significant morphological changes of CHO cells. The culture supernatant of *S.dysenteriae* RIMD 3101001, used to isolate the CHO-factor as described in the preceding section, caused only 1.3% morphological change. This is consistent with the finding that crude preparations did not cause any morphological changes of CHO cells, but column chromatography of the preparations showed that they contained activity (21).

Since mitomycin C stimulates the production of heat-labile enterotoxin of enterotoxigenic *Escherichia coli* (22, 23), the effect of mitomycin C on the production of the CHO-factor by these 39 strains of *Shigella* was studied. With some strains, production of the CHO-factor was significantly increased by addition of mitomycin C to the medium (Table 4). In this experiment the cells were cultured in brain heart infusion broth, mitomycin C (1 μg/ml) was added in the early logarithmic growth phase, and incubation was continued for about 20 hours. Not only *S.dysenteriae*, but also other species of *Shigella* produce the CHO-factor. From these results, it is concluded that all species of *Shigella* produce the factor that causes morphological changes of CHO cells.

TABLE 4

EFFECT OF MITOMYCIN C ON THE ACTIVITY OF CULTURE SUPERNATANTS OF VARIOUS *SHIGELLA* STRAINS TO CAUSE MORPHOLOGICAL CHANGES OF CHO CELLS

Strain	% Elongation	
	-Mitomycin C	+Mitomycin C
S. dysenteriae		
RIMD3101018	1.8 ± 0.1	18.3 ± 0.9
RIMD3101021	1.5 ± 0.3	11.5 ± 0.6
RIMD3101013	2.0 ± 0.6	13.1 ± 0.9
RIMD3101014	3.1 ± 0.4	17.7 ± 1.6
RIMD3101010	3.7 ± 0.7	16.6 ± 0.9
RIMD3101001	1.3 ± 0.3	2.5 ± 0.4
RIMD3101005	10.4 ± 1.4	19.3 ± 2.7
RIMD3101003	11.8 ± 1.1	16.8 ± 1.3
S. flexneri		
RIMD3102018	2.0 ± 0.1	13.5 ± 1.3
RIMD3102034	3.4 ± 1.2	16.3 ± 1.9
RIMD3102017	3.6 ± 1.0	16.6 ± 2.5
RIMD3102016	3.8 ± 0.2	13.3 ± 1.1
RIMD3102002	3.3 ± 0.8	10.4 ± 0.4
RIMD3102015	12.5 ± 1.1	15.0 ± 1.4
S. boydii		
RIMD1998003	5.7 ± 0.4	16.8 ± 0.8
S. sonnei		
RIMD3104007	2.0 ± 0.3	18.7 ± 0.9

ACKNOWLEDGEMENT

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Chapter 15

Isolation, Purification and Characterization of a *Shigella* Phage

M.I. Huq and M.A. Salek

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

ABSTRACT

Of the typing methods of bacteria used for the epidemiological investigation of the disease, bacteriophage typing appears to be the most sensitive. Many authors have published papers on the use of cholera phage in the epidemiological studies of cholera and differentiation of biotypes of *V.cholerae*. Studies on *Shigella* phages are limited to *S.flexneri* and *S.sonnei* phages. During the routine research for bacteriophage from stools of the patients or bacterial cultures from them we isolated a phage which lysed *Shigella* flexneri on the isolation plate. The phage was purified and its properties studied. It produces big round plaque, has a burst time of about 18-22 minutes, is neutralized by its homologous antiphage serum and lyses all the *S.dysenteriae* type 1 and all the *S.flexneri* type 2, part of type 3 and 4 and none of type 1, 5 and 6. It does not attack any of the other *Shigella* sp. *Salmonella*, *E.coli*, Paracolon, Proteus and *V.cholerae* or other vibrios and Aeromonas sp.

INTRODUCTION

Bacteriophages are a group of bacteria-specific viruses which have the ability to bring about lysis of growing bacterial cultures. Since 1917 when d'Herelle published his independent discovery of bacteriophage, interest on the phages grew (1). d'Herelle showed that when the filtrate of the stool from a patient was added to a growing *Shiga bacillus* the growing culture was killed and there was an increase in the titre of the lytic principle in the broth. This behaviour was suggested by him to be consistent with an ultra virus, pathogenic for bacteria, destroying its host cells as it multiplies. Because of the susceptibility of pathogenic bacteria to phages, and because of the wide distribution of phages in nature, d'Herelle suspected that they played a role in resistance to and recovery from the disease. The role of bacteriophage in the characterization of bacteria and their genetic behaviour have been studied by many authors. Anderson *et al* (1956) worked on the bacteria phage typing of enteric pathogens, and showed clearly that the typing pattern is of use in the epidemiological investigation of the disease.

Most of the published work in South Asia has been on cholera phage, and results of studies with many epidemic strains have been reported (2,3). Publications on phages acting on *Shigella* are very limited. During the late 1960's a few studies were done on the use of bacteriophage and colicines for typing *Shigella*, especially *S.flexneri*. Stefan Slopek and his associates in Wrockland, Poland (4) divided 767 strains of *Shigella flexneri* into 69 types using 12 dysentery bacteriophages. Vera Lazlo *et al* (5) in Budapest worked on the phage typing of *Shigella flexneri*, and got 90 phage types for 4606 *S. flexneri* strains. In their opinion, phage typing proved to be suitable in epidemiological practice for subdividing the serotypes, for tracing the infection routes and for distinguishing between cases from different foci (6). Bruneda and Farmer (7), while trying to establish a standardized scheme for differentiation of *Shigella* strains, typed 265 strains of *Shigella sonnei* into 87 different lysis patterns. Work reported so far has been limited to typing a single serotype of *Shigella* by clusters of phages.

In the microbiology branch of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) we isolate every year large number of *Shigella* from dysentery patients reporting to the hospital. We sought a good classifying set of phage for *Shigella*. New isolates of phage from stools of patients reporting to our hospital were tested against a panel of *Shigella* organisms of all serotypes, to observe the lytic patterns. In the course of the search for new phages, we isolated from a culture plate of patient No. OPD-153950 a phage which caused lysis to the pure growth of *S.flexneri* on the plate. The phage plaque was picked up, purified and found to attack a wide range of *Shigella* but not other bacteria.

MATERIALS AND METHODS

Media used: for all phage studies we initially used two different broth and plating media. When Trypticase Soy Broth (TSB) BBL was used as enriching media, the plating media was Trypticase Soy Agar (TSA), BBL and when T_1N_1 (Trypticase 1%, sodium chloride 1%, pH 7.2) broth was used as enriching media the plating media was T_1N_1 Agar. On comparison, it was found that T_1N_1 media, used in our laboratory for Cholera phage work, gave comparable results to Trypticase Soy medium. Hence, all work was carried out in T_1N_1 medium.

ISOLATION OF PHAGE PREPARATION

The host culture No.P-18167 was inoculated into 5 ml T_1N_1 broth, and incubated for 4-6 hours until visible turbidity was present. One defined isolated plaque from the isolation plate was inoculated into a growing culture in liquid medium and incubated further for 6-8 hours at 37°C, and kept overnight at room temperature. The residual bacteria in the phage-enriched broth were removed by filtering the broth through 0.45 millipore filter. The titre of the enriched preparation of phage was enumerated by the following methods:

a) Miles and Misra method: Making serial 10-fold dilution of the phage broth and dropping the phage dilution onto a lawn made of 6 hours grown culture of the host strain and incubating overnight at 37°C. The lysis in the area of the drop were recorded as Confluent lysis (CL), Semiconfluent lysis (SCL), 3+, 2+, 1+. The count of phage plaque on the last countable dilution may be taken to quantitative the phage titre in the preparation.

b) Agar overlay method: 0.1 ml of the respective 10-fold dilution of the phage preparation was mixed with 0.5 ml of the 6 hour grown culture and the mixture was put in a tube containing 3.5 ml of the 1/2 strength T_1N_1 broth containing 0.75% of Agar kept at 45°C. The whole mixture was mixed well and poured onto well dried T_1N_1 Agar plate. After the media were set the plates were incubated for 24 hours and read. The total number of plaques in the highest dilution was taken to quantitate the phage titre in the preparation (8). From the plates, the size and shape of the plaques were recorded.

PURIFICATION OF PHAGE PREPARATION

Method (b) described above was used to purify the phage preparation. A single plaque of the type seen on the plate was picked up and enriched again as per above method. The bacterial culture was picked up and enriched, again as per above method. The bacterial culture picked along with the plaque acted as the enrichment culture. The method was repeated to get a homogeneous plaque type in the phage preparation.

Determination of Burst Time.

Overnight grown culturesof p-18167 were inoculated into 5 ml. T_1N_1 broth and incubated at 35°C in a well-controlled water bath. Bacterial count was made at 0 hrs. from the inoculated tube and then at minute 10, 15, 20 etc. up to 75 minutes, at 5 minute intervals. Serial tenfold dilution of the broth culture was made, and appropriate dilutions were plated onto T_1N_1 Agar plate to get countable colony. The total number of colonies was recorded and burst time of the bacteria calculated.

Preparation of Antiphage Serum.

Antiphage serum was prepared by immunizing rabbits weighing 1-2 kg. Four rabbits in individual cages were kept aside for 1 week, before starting the injections. Three I.V. injections of 0.3 ml, 0.4 ml, and 0.6 ml of pure phage preparation of titre 109 pfu/ml were given on alternate days. After one week, test blood was drawn. As the titre was not very high, 3 more injections of 0.5 ml, 0.7 ml and 1.0 ml of the above phage were given on every alternate day, and test bleeding done after 1 week's rest. The antiphage titre of serum was found sufficiently high and all the rabbits were bled to death using aseptic precautions. After separation, the serum was kept at 4°C without preservative.

The ability of the serum to neutralize the phage preparation was measured using serum diluted to 1/10, 1/100 and 1/1000 and known amount of phage by using methods used by Islam (9). The titration of phage from phage serum mixture in each tube was made at 5, 15, 30, 45, 60, 75 and 90 minutes, by using Agar overlay method.

The lytic pattern of this phage against other members of enterobacteriaceae, *V.cholerae*, pseudomonas and aeromonas was observed by dropping the dilutions of the phage on the lawn of respective cultures on T_1N_1 agar plates. Clear visible lysis of the bacteria by the phage preparation forming distinct plaques with diluted phage, was recorded.

RESULTS

Figure 1 shows the multiplication pattern of the phage preparation when added in the broth in presence of the homologous bacteria. The first bursting took place within 18-22 minutes; the second within 35-40 minutes, and the third within 54-58 minutes.

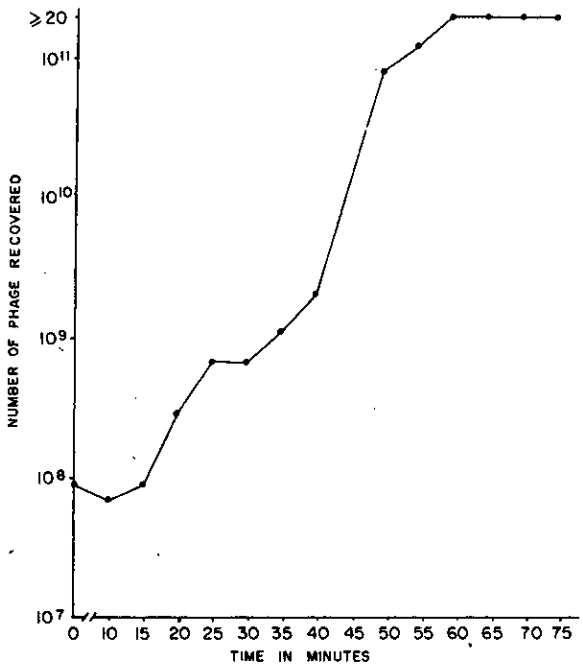

Fig. 1: Multiplication pattern of the phage preparation

Table 1 shows the results of a neutralization test done with the antiphage serum and different dilutions of phage at different intervals.

Table 2 shows the lytic pattern of the phage when tested against other Enterobacteriaceae. The phage attacked all *S. dysenteriae* type 1, all *S.flexneri* type 2 and some type 3 and 4, but not type 1, 5 and 6. None of the other *Shigella* serotypes, *Salmonella, E.coli,* Para colon, proteus, *V.cholerae* and Aeromonas sp. were attacked.

DISCUSSION

The use of phage in typing bacterial strains to define epidemiological patterns of the disease has been documented previously (2). Recent work by Slopek *et al* has indicated that bacteriophage typing could become the method of choice for studying outbreaks from *S.sonnei*. Most later work was done on cholera phages which have been characterized and patterns well established. The work on *Shigella* phage was mainly limited to the phages active against *S.flexneri* and *S.sonnei*. We hoped to find a universal phage which would attack the important prevalent *Shigella* species, and carried out a routine search for such phages. The phage reported in this paper was found to have wide-ranging lytic activity. With the usual enrichment procedure, using plain Trypticase broth, we could enrich the phage to 2.1×10^{10} pfu/ml, which titre is similar to results obtained with *E.coli* phages but different from other *Shigella* phages. This phage preparation did not lose its titre appreciably when kept at 4°C for about 2 months. They form distinct round crenated plaques with a second peripheral zone of lysis.

TABLE 1

NEUTRALISATION OF PHAGE BY ANTIPHAGE SERUM

Serum dilution	1/10		1/100		1/1000	
Expected No. of Phage in the mixture	160	1600	160	1600	160	1600
Duration of incubation	Observed		p.f.u.	per plate		
5 mins	18	270	74	SCL	SCL	CL
15 "	2	72	21	SCL	CL	CL
30 "	0	11	9	>SCL	SCL	CL
45 "	0	0	0	SCL	SCL	CL
60 "	0	0	0	>1000	SCL	CL
75 "	0	0	0	560	SCL	CL
90 "	0	0	0	410	CL	CL

p.f.u. = Plaque-forming unit

SCL = Semi-confluent lysis

CL = Confluent lysis

As *S. shiga* and *S. flexneri* type 2, 4 and 6 constitute almost 75-80 percent of the *Shigella* isolates in our laboratory we feel strongly that biochemically confirmed *Shigella* sp. may be further confirmed by testing them with this phage preparation, thus avoiding in most cases the use of very costly antisera imported from abroad.

TABLE 2

RESULTS OF LYTIC PATTERN OF *SHIGELLA* PHAGE AGAINST VARIOUS TYPES AND ENTEROBACTERIACEAE *V.CHOLERAE*, AND OTHER GRAM-NEGATIVE BACTERIA

Name of the bacteria	No. tested	Lytic pattern
Shigella dys. type 1 *(Shigella shiga)*	171	All attacked
Shigella dys. type 2	22	None attacked
Shigella dys. type (3-10)	11	None attacked
Shigella flexneri type 1	24	None attacked
type 2	31	All attacked
type 3	23	Attacked
	15	Not attacked
type 4	16	Not attacked
	17	
type 5	12	Not attacked
type 6	8	Not attacked
Shigella boydii	20	Not attacked
Shigella sonnei		
Phage 1	16	Not attacked
Phage 2	18	Not attacked
Salmonella	49	Not attacked
E.coli	52	Not attacked
El Tor Vibrio	73	Not attacked
NAG vibrio	39	Not attacked
Paracolon	24	Not attacked
Aeromonas	8	Not attacked
Proteus	14	Not attacked

Considering the lytic pattern of the phage, as well as the high stable titre that can be obtained, we are planning a study of treating the *Shigella* patients with high oral dosage of this phage in the same way cholera phage has been used in the prophylaxis and treatment of cholera (10,11). The

effectiveness of the phage therapy will be determined by the reduction of stool output, and duration of stool positivity, as well as the drop of *Shigella* count in the stool. Initially we plan to induce dysentery in monkeys or rabbits with *Shigella* followed by treatment with the phage.

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Chapter 16

The Leukemoid Reaction in Shigellosis

Thomas C. Butler, M.R. Islam and P.K. Bardhan

International Centre for Diarrhoeal Disease Research, Bangladesh,Dhaka, Bangladesh

ABSTRACT

To examine the clinical significance of the leukemoid reaction in shigellosis, charts of 3,573 hospitalized patients with faecal cultures positive for *Shigella* species were reviewed. There were 136 cases (3.8%) of leukemoid reactions, defined as >50,000 WBC per mm^3. Sixty-eight percent of patients with leukemoid reactions were less than 4 years old. When compared to patients without leukemoid reactions, the leukemoid reactions were significantly associated with children aged less than 4 years and less than 10 years ($p<0.005$). The most common species of *Shigella* associated with the leukemoid reactions was *S.dysenteriae* 1, isolated from 96 patients (71%), whereas, the most common species in patients without leukemoid reactions was *S.flexneri*, isolated from 2,119 patients, or 62% ($p<0.05$). The mortality rate in patients with the leukemoid reactions was 22%, compared to 7.4% in patients without leukemoid reactions ($p<0.001$). These findings indicate that in patients with shigellosis, the leukemoid reaction was significantly associated with young children, isolation of *S.dysenteriae* 1, and increased mortality.

INTRODUCTION

The pathogenesis of shigellosis, following ingestion of bacteria, requires the invasion of intestinal epithelial cells by bacteria and multiplication of bacteria. The resulting fever, diarrhoea, and dysentery are caused by the host response to bacterial products, which may include endotoxin and an enterotoxin (1-4).

In 1975, Rahaman *et al* (5) described the association of dysentery caused by *Shigella dysenteriae* 1 and the leukemoid reaction in Bangladesh. Fifty-one patients with shigellosis in this report had white blood cell counts greater than 50,000 per mm^3, and 44 of these patients were infected with *S.dysenteriae* 1. Children were mostly affected by the leukemoid reactions, which were characterized by granulocytic predominance and shifts toward immature granulocytes. The reactions occurred late in the course of the dysentery after intestinal symptoms were subsiding. Hemolysis with red blood cell fragmentation was common. Some of the patients in the report of Rahaman *et al* developed renal failure. A subsequent study of Koster *et al* (6) in Bangladesh described the hemolytic-uremic syndrome associated with shigellosis and leukemoid reactions; and suggested that endotoxin entered the blood from the inflamed colon and initiated intravascular coagulation in the blood vessels of the kidney, leading to renal cortical necrosis.

The present study seeks to describe more fully the association of the leukemoid reaction with acute shigellosis. The results indicated that young children were preferentially affected, that *S.dysenteriae* 1 frequently caused the leukemoid reaction, and that the leukemoid reaction was a prognostic factor for high mortality.

MATERIALS AND METHODS

At the ICDDR,B (Formerly the Cholera Research Laboratory) patients with diarrhoea are screened for either admission or outpatient treatment. Patients who appear severely ill or have complications, such as malnutrition, seizures, or pneumonia, are admitted. All admitted patients undergo further testing for faecal culture and complete blood count. In this study we obtained the list of all 3,573 admitted patients from July 1975 to June 1980 whose faecal cultures were positive for *Shigella* species. Fresh faecal specimens or rectal swabs had been received in the Microbiology Laboratory and streaked onto Salmonella-Shigella agar and MacConkey agar. After overnight incubation at 35°C, selected colonies that were non-lactose fermenting were subcultured onto Kligler-iron agar and motility-indole-urea agar. Those isolates showing reactions consistent with *Shigella* species were confirmed and speciated by polyvalent and monovalent agglutinating sera (Wellcome Diagnostics, Dartford, England). The charts of all patients were obtained and the white blood cell counts on the day of admission and on subsequent measurements were recorded.

Patients with one or more WBC counts ⩾ 50,000 per mm^3 were considered to have the leukemoid reaction, and patients with WBC of <50,000 per mm^3 were counted as non-leukemoid cases.

From all charts, the following data were recorded: age, sex, *Shigella* species isolated, and clinical outcome. From the charts of patients with the leukemoid reaction, further clinical and laboratory information relating to the hemolytic-uremic syndrome was obtained. Hemolysis was defined as a decrease in the hematocrit of ⩾ 10% from the time of admission to a subsequent measurement. Uremia was defined as a blood urea nitrogen concentration of ⩾ 60 mg per 100 ml or a serum creatinine of ⩾ 2 mg per 100 ml. The hemolytic-uremic syndrome was defined as the presence of both hemolysis and uremia.

RESULTS

Incidence and age distribution of the leukemoid reaction in patients with shigellosis.

Hospital charts of 3,573 patients with faecal cultures positive for *Shigella* species were reviewed. The admission white blood cell count (WBC) was measured for all patients, and those with WBC ⩾ 50,000 per mm^3 were considered to have leukemoid reaction. The incidence of leukemoid reactions was seen in 136 patients out of the 3,573 hospitalized shigellosis cases (3.8%).

Shigellosis occurred predominantly in young children. The age distributions of the 3,437 patients without leukemoid reactions and the 136 patients with leukemoid reactions are shown in Table 1. A comparison of the two revealed that the leukemoid reaction was more strongly associated with ages less than 4 years and less than 10 years than was shigellosis without the leukemoid reaction ($p<0.005$ by Chi-square test). The predilection of the leukemoid reaction to occur in young children was not apparent in the first year of life, but was manifest in all childhood years from 1-to-10 except for one (Table 1).

Sex distribution of patients with shigellosis.

Of the patients without leukemoid reactions, 2,026 (59%) were males. Of the 136 patients with leukemoid reactions, 72 (53%) were males, or 53%. This preponderance of male patients with shigellosis was not significantly different between the patients with and without leukemoid reactions ($p>0.05$).

TABLE 1

AGE DISTRIBUTION OF PATIENTS WITH SHIGELLOSIS WHO PRESENTED WITHOUT LEUKEMOID REACTIONS OR WITH LEUKEMOID REACTIONS

Age in Years	Non-Leukemoid (N=3437) No.	Percent*	Leukemoid Reaction (N=136) No.	Percent*
0-.9	643	(19)	25	(18)
1-1.9	499	(15)	23	(17)
2-2.9	425	(12)	26	(19)
3-3.9	342	(10)	19	(14)**
4-4.9	147	(4.3)	7	(5.1)
5-5.9	142	(4.1)	9	(6.6)
6-6.9	102	(3.0)	7	(5.1)
7-7.9	74	(2.1)	2	(1.5)
8-8.9	63	(1.8)	4	(2.9)
9-9.9	51	(1.5)	6	(4.4)
≥ 10	949	(28)	8	(5.9)**

* Percent of all cases with non-leukemoid or leukemoid reaction in each age group.

** Association of leukemoid reactions with ages <4 yr and <10 yr significant, respectively, $\chi^2 = 8.8$ and 31.5, $p<0.005$.

Hematological features of the leukemoid reaction in shigellosis.

The WBC counts in the 136 patients with leukemoid reactions ranged from 50,000 to 195,000 per mm^3 with a mean of 66,300 per mm^3. The differential WBC counts showed that most patients had neutrophilia with increased numbers of immature neutrophils, including bands, metamyelocytes, and myelocytes. The mean percentages of each kind of white cell were calculated: polymorphonuclear

leukocytes 45%, immature forms 29%, lymphocytes 20%, monocytes 2%, and eosinophils 1%. This distribution of white cells indicates that the greatest increases in absolute numbers of white cells in peripheral blood were in polymorphonuclear leukocytes and immature granulocytes, but that the absolute numbers of lymphocytes and eosinophils were also increased.

Shigella species isolated from patients with leukemoid reactions.

The most frequently isolated species in patients without leukemoid reactions was *S. flexneri*, which was isolated from 62% of the patients (Figure 1) The second most frequent isolate was *S. dysenteriae* 1, isolated in 20%.

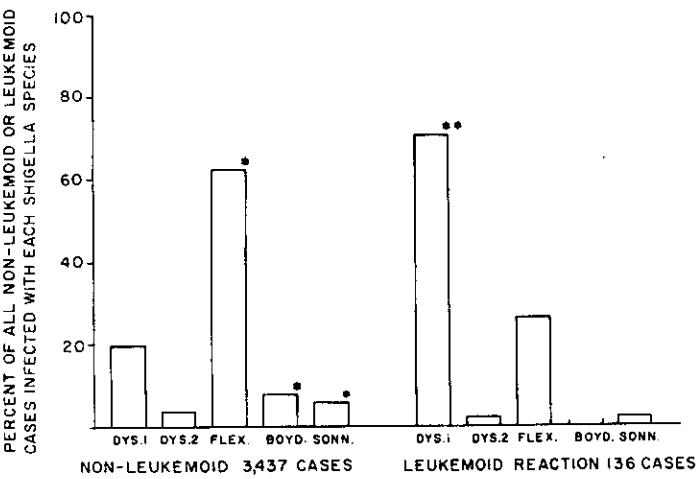

Fig. 1: Distribution of *Shigella* species isolated from patients without the leukemoid reaction and with leukemoid reactions.

* Isolations of *S. flexneri*, *S. boydii*, and *S. sonnei* were associated with non-leukemoid cases ($p<0.05$).

** Isolation of *S. dysenteriae* 1 was associated with leukemoid reactions ($p<0.001$).

On the other hand, *S. dysenteriae* 1 was the most frequent species in patients with leukemoid reactions, isolated from 71% of patients, and *S. flexneri* was isolated from only 26% of these patients. By Chi-square analysis each *Shigella* species was examined for its association with non-leukemoid or leukemoid cases. Significant associations were found for *S. flexneri*, *S. boydii*, and *S. sonnei* with the non-leukemoid cases and for *S. dysenteriae* 1 with the leukemoid cases (<0.05).

The age distribution of infections with *S. flexneri* and *S. dysenteriae* 1 were examined in patients without and with leukemoid reactions, to determine whether the association of infection with *S. dysenteriae* 1 with leukemoid reactions was a possible result of *S. Dysenteriae* 1 attacking preferentially younger children. In Table 2, the age distribution of these two Shigella species shows that patients without leukemoid reactions were predominantly infected with *S. flexneri* in all age groups. Patients with leukemoid reactions were infected predominantly with *S. dysenteriae* 1, and this pattern was reflected in every year of life, as well as in the group $\geq$ 10 years old.

TABLE 2

DISTRIBUTION OF *SHIGELLA* SPECIES BY AGE IN PATIENTS WITHOUT AND WITH LEUKEMOID REACTIONS

	Non-Leukemoid		Leukemoid Reactions	
Age in years	No. of *S. dysenteriae* 1	No. of *S. flexneri*	No. of *S. dysenteriae*	No. of *S. flexneri*
0-0.9	56	459	14	9
1-1.9	84	348	18	5
2-2.9	94	260	16	10
3-3.9	71	195	11	5
4-4.9	28	92	8	0
5-5.9	38	85	6	3
6-6.9	27	57	5	2
7-7.9	18	32	1	0
8-8.9	17	37	5	0
9-9.9	13	31	4	1
≥ 10	229	523	8	0

Mortality of shigellosis related to the leukemoid reaction.

In the 136 patients with leukemoid reactions, 28 patients died in the hospital, giving a mortality rate of 21%. In the 3,437 patients with shigellosis without leukemoid reactions, there were 254 deaths, or 7.4% mortality. The increased mortality rate of shigellosis with the leukemoid reactions was statistically significant by Chi-square testing ($p<0.001$). Examination of mortality by age was carried out and the results displayed in Figure 2. During infancy (the 0-0.9-year group) the mortality rates were higher than for older age groups combined, for both non-leukemoid and leukemoid groups ($p<0.05$). In the 0-0.9-year age group, the mortality rate in patients with leukemoid reactions, 36%, was significantly higher than the mortality of patients without the leukemoid reaction, 12% ($p<0.05$). Thus, the greater mortality associated with the leukemoid reaction affected all

age groups, including infancy and the over 10 years group, although there were too few patients or no deaths in some age groups of the leukemoid reactions to measure this effect (Figure 2).

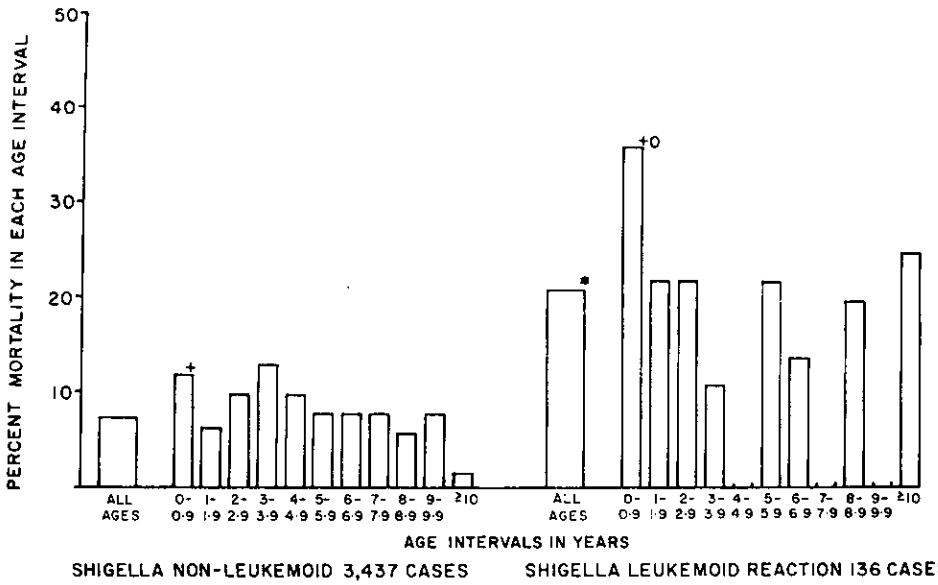

Fig. 2: Mortality rates by age in patients with shigellosis, both without leukemoid reactions and with leukemoid reactions.

* For all ages, the mortality was significantly higher in leukemoid than non-leukemoid cases ($p<0.001$).

\+ Mortality rates in the 0-0.9-year age group higher than in older age groups, for both leukemoid and non-leukemoid groups ($p<0.05$). The mortality rate in the 0-0.9-group was higher in leukemoid reactions than in non-leukemoid cases ($p<0.05$).

To determine whether the species of *Shigella* may have affected mortality separately, the mortality rates were examined by species (Table 3). The increased mortality in patients with leukemoid reactions was not enhanced further in those infected with *S.dysenteriae* 1. Actually, the patients infected with *S.flexneri* had a higher mortality rate (29%), but this was not significantly different from the mortality of those infected with *S. dysenteriae* 1 ($p>0.05$). Similarly, the mortality rate in patients without leukemoid reactions did not appear to be influenced by the infecting species.

The mode of death in patients with the leukemoid reaction was assessed by following hemolytic anemia and renal failure, because of the known association of the hemolytic-uremic syndrome with shigellosis in Bangladesh. Table 4 shows that 39 patients developed the hemolytic-uremic syndrome and a further 47 developed hemolysis or uremia alone. The mortality rates of

TABLE 3

MORTALITY BY *SHIGELLA* SPECIES ISOLATED FROM PATIENTS WITHOUT LEUKEMOID REACTIONS AND WITH LEUKEMOID REACTIONS

	Non-Leukemoid (N=3437)			Leukemoid Reaction (N=136)		
	No.of cases	No. of Deaths	% (Mortality)	No. of Cases	No. of Deaths	% (Mortality)
S.dysenteriae 1	675	28	(4.1)	96	17	(18)
S.dysenteriae 2	145	11	(7.6)	3	0	(0)
S.flexneri	2119	164	(7.7)	35	10	(29)
S.boydii	282	24	(8.2)	0	0	(0)
S.sonnei	216	26	(12)	2	1	(50)
Total	3437	253	(7.4)	136	28	(21)

patients with these different complications were not significantly higher than for patients without these complications. Thus, the hemolytic and uremic complications are only part of the pathophysiology of the fatal outcome in patients with shigellosis and the leukemoid reaction.

DISCUSSION

In the developing countries of the Indian Subcontinent, shigellosis can be a serious disease, and carries a higher mortality rate than in developed countries. The present study reviewed the case records of 3,573 hospitalized patients with culture-proven shigellosis at a hospital for diarrhoeal diseases in Bangladesh during a five-year period. Leukemoid reaction was not a rare complication in these seriously ill patients with shigellosis.

Most of the hospitalized patients with shigellosis, both with and without leukemoid reactions, were young children less than 4 years old. The leukemoid reactions, however, were distributed even more than non-leukemoid cases into the early years of life. Eighteen per cent of the leukemoid reactions were

TABLE 4

FREQUENCY AND MORTALITY OF HEMOLYTIC AND UREMIC COMPLICATIONS IN SHIGELLOSIS PATIENTS WITH LEUKEMOID REACTIONS*

	No. Cases	No. Deaths	(% Mortality)
All Leukemoids	136	28	(21)
Hemolytic-Uremic Syndrome	39	8	(21)
Hemolysis only	42	10	(24)
Uremic only	5	1	(20)
No. Complication	50	9	(18)

* Hemolysis was defined as a decrease of the hematocrit by ≥10% between two measurements. Uremia was defined as serum urea nitrogen ≥ 60 mg per 100 ml or serum creatinine ≥2 mg per 100 ml. Hemolytic-uremic syndrome was defined as both hemolysis and uremia, as above.

observed in infants (0-0.9-yr), but a similar percentage of non-leukemoid cases were also infants. The predilection for leukemoid reactions to occur in early childhood became manifest in the second year of life and continued until 10 years of age. Leukemoid reactions caused by other acute bacterial infections also occur in children (7); and this may be explained, in part, by the immaturity of the bone marrow in early age, or lack of immunity that permits the infection to progress more fulminantly than in older patients.

Although the majority of all patients with Shigella infection admitted to our hospital were infected with *S. flexneri*, 71% of the leukemoid reactions occurred in patients infected with *S. dysenteriae* 1. This strong association of the leukemoid reaction in shigellosis with *S. dysenteriae* 1 confirms the earlier observations of Rahaman *et al* (5). *S. flexneri* infections did result in 25% of the leukemoid reactions, but *S. flexneri* was significantly less likely to cause leukemoid reactions than *S. dysenteriae* 1 ($p<0.05$). In patients of all age groups, *S. dysenteriae* 1 infections predominated over *S. flexneri* infections in patients with leukemoid reactions, whereas, *S. flexneri* predominated in patients without leukemoid reactions. Thus, there must be a biological feature of *S. dysenteriae* 1 that confers on the bacterium the preferential ability to evoke leukemoid reactions in infected hosts. Endotoxin is one component

of Gram-negative bacteria that is capable of stimulating the bone marrow to increase production of white blood cells. Furthermore, endotoxemia has been detected by Koster *et al* (6) in some patients with shigellosis and leukemoid reactions. It is possible that the endotoxin of *S.dysenteriae* 1 is qualitatively more potent than the endotoxin of *S.flexneri* in stimulating the bone marrow to produce leukemoid reactions. Alternatively, *S.dysenteriae* 1 infections may produce more severe colitis, and this allows more endotoxin to enter the circulation.

The leukemoid reactions in this series of patients were mainly granulocytic and characterized by increased number of band forms, metamyelocytes, and myelocytes. The granulocytic nature of the leukemoid reactions in shigellosis is consistent with previous reports from Bangladesh (5,6). In the United States, however, Barrett-Connor reviewed 187 cases of shigellosis in which the highest white blood cell count was 43,000 per mm^3 with a predominance of lymphocytes (8). Actually, the lymphocytes in many of our patients were also increased and the mean absolute lymphocyte count was distinctly higher than normal. The preponderance of granulocytes in the peripheral blood is consistent with the acute bacterial infection of the colonic mucosa, which produces a polymorphonuclear infiltration of the mucosa, ulceration, crypt abscesses, and the outflow of polymorphonuclear leukocytes into the diarrhoeal stool. This latter feature of shigellosis is used as a clinical test for faecal leukocytes for the purpose of making an early diagnosis of the disease.

The mortality rate of our Shigella-infected patients with leukemoid reactions was 21%, compared to 7.4% for patients without leukemoid reactions ($p<0.05$). Age specific-mortality rates indicated that the highest rates were in infancy both in leukemoid and non-leukemoid cases, but the increased mortality rates of the patients with leukemoid reactions was observed in all age groups.

Although *S.dysenteriae* 1 infection caused the majority of the leukemoid reactions, the mortality rate in the patients with leukemoid reactions was not influenced by the infecting species of *Shigella*. Actually, the mortality rates in patients infected with *S.flexneri*, both in the leukemoid and non-leukemoid groups, were higher than the mortality rates in patients with *S.dysenteriae* 1 infections, but these differences were not statistically significant.

The hemolytic and uremic complications of shigellosis with the leukemoid reaction, which has been described already in Bangladesh (5,6), occurred also in most of our patients. The mortality rates, however, did not appear to be influenced by the presence of these complications. Actually, 50 cases out of the 136 leukemoid reactions did not have the hemolytic or uremic complications, but still showed a mortality rate of 18%. Therefore, there must be other pathophysiological effects of the leukemoid reactions that may lead also to fatal outcomes in these patients.

Our results thus indicate that the leukemoid reaction in shigellosis is itself a bad prognostic sign. Other workers investigating prognostic factors in shigellosis have reached different conclusions. Barrett-Connor found that the WBC had no influence on severity in the USA, except that patients with seizures tended to show leukocytosis (8). The presence of *Shigella* sepsis does appear to increase mortality. Duncan *et al* (9) in the USA reported that 4 of 8 septicemic patients died. These patients were infected with *S.flexneri* and were less than 3 years old. Compared to surviving patients, the fatal cases more frequently were dehydrated, afebrile, leukopenic, hypoalbuminemic, had blood in stools, and were infected with ampicillin-resistant organisms. The leukopenia in the patients described by Duncan *et al* (9) contrasts with the leukemoid reactions in our fatal cases, but the differences could be due to the *S.flexneri* infections and that they were bacteremic. In our patients blood cultures were not regularly obtained and the frequency of sepsis could not be determined. In South Africa, Scragg *et al* (10) reported also that *Shigella* sepsis in children was associated with a high mortality rate of 45%, compared to 27% in all *Shigella* infections. In this report, bad prognostic factors were malnutrition and infancy, as the median age of fatal cases was 10 months. In India, Koshi *et al* (11) reported fatalities in 6 of 7 cases of sepsis with *S.dysenteriae* 1. None of the patients had leukemoid reactions,but 2 of the fatal cases had WBC counts of 30,000-40,000 per mm^3. The usefulness of our results is that clinicians can use the WBC as one prognostic indicator in patients with shigellosis, especially in geographic areas where *S. dysenteriae* 1 infections are endemic. Furthermore, our findings in the leukemoid reaction should stimulate the search for factors in *Shigella* infection that cause the leukemoid reaction and how white cell products may play a role in the pathophysiology of shigellosis.

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Chapter 17

Comparative Treatment of Shigellosis with Trimethoprim-Sulphamethoxazole and Ampicillin

Md. Yunus, A.S.M. Mizanur Rahman, A.S.G. Faruque and Roger I. Glass

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

ABSTRACT

Following a nationwide outbreak of *Shigella* dysentery type 1 resistant to ampicillin, the drug of choice, a clinical trial was conducted to compare the efficacy of trimethoprim-sulfamethoxazole vs ampicillin for the treatment of *Shigella* dysentery. Patients with symptoms of dysentery and no other complicating illness were randomized into two groups. Patients in the two groups were comparable at the time of hospital admission with regard to age, sex, presenting complaints and *Shigella* strains. They responded well with both regimens, and there was no significant difference in the length of time until stool became culture-negative (1.4 days), temperatures returned to normal (2.7 days) and faecal WBCs disappeared (3.0 days). Abdominal pain, tenesmus and stool blood and mucous improved significantly faster with trimethoprim-sulfamethoxazole than with ampicillin. There was no evidence of toxicity with either drug. While both drugs are effective for the treatment of *Shigella* dysentery, trimethoprim-sulfamethoxazole was considered to be superior.

INTRODUCTION

Shigellosis is a major health problem in Bangladesh and other developing countries (1,2,3). A recent nationwide outbreak of *Sh.dysenteriae* type 1, or *Shiga bacillus*, resulted in many deaths and complications (4,5). During and

after this outbreak, resistance of *Shigella*, particularly *S.dysenteriae* type 1, to common antibiotics was first noted in Bangladesh (6). Mutanda in 1981 found that 6% of the 87 *Shigella* strains isolated in 1979 were resistant to ampicillin and that 6 were resistant to several antibiotics (7). Such resistance has occurred elsewhere to most safe and useful agents. In the United States, for example, 17% of *Sh.flexneri* and 45% of *Sh.sonnei* in 1974 were resistant to ampicillin (8). The appearance of ampicillin resistance in Bangladesh and the high prevalence of skin rash with use of this drug has given rise to the need for alternative antibiotics. A study was conducted to compare trimethoprim-sulfamethoxazole and ampicillin for the treatment of *Shigella* dysentery in rural Bangladesh.

SUBJECT AND MATERIALS

The study was performed during 1977-1979 at the rural treatment centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (formerly the Cholera Research Laboratory) located in Matlab thana, Comilla district, about 45 Km Southeast of Dhaka. This centre, in operation since 1963, provides diarrhoeal treatment services to 4000-6000 patients per year from a well-defined study area. About 6% of the patients reporting to the rural treatment centre have shigellosis. Details of the study area, its people and field research procedures have been reported (9).

Patients with symptoms of dysentery, abdominal pain, tenesmus, and clinically suspected of having *Shigella* were considered for the study. We excluded pregnant women, children below three months of age, patients with a history of adverse reaction to penicillin or sulfanomide, or a complicating illness pneumonia or a history of prior treatment. Patients meeting the criteria and willing to participate (guardians in case of minors) were assigned to a treatment group at random.

A full clinical examination of the patient was done after admission. Before therapy, stool specimens for microscopic examination, rectal swabs for culture, urine for analysis, and blood for white blood cell, differential and platelet counts were collected. Trimethoprim-sulfamethoxazole (tablet or suspension) was administered 6 mg/kg/day, 12 hourly for 5 days, or ampicillin was given 50 mg/kg/day, six hourly divided doses to patients weighing 15 kg or more and in double dose for smaller children. Stool was cultured on taurocholate-tellurite-gelatin agar, shigella-salmonella agar, and MacConkey agar plates daily, until these were negative for three consecutive days.

Patients whose stool yielded *Salmonella* sp. or *V.cholerae* O1 were dropped from the study. Daily stool microscopy was performed for three consecutive days, to exclude vegetative amoeba and giardia and to determine when the stool became free of leukocytes (<10 leukocytes/hpf). *Shigella* isolates were typed with specific antisera; and were tested for antibiotic resistance to

trimethoprim-sulfamethoxazole, ampicillin, tetracycline, streptomycin, chloramphenicol, gentamycin, and kanamycin using the Bauer-Kirby (10) technique. Oral rehydration solution or intravenous acetate solution was given as required, intake and output was measured every 8 hours, and vital signs were recorded every 4 hours. No other medication was given. The stool characteristics (volume, consistency, presence of blood and mucous), fever, presence of abdominal pain, tenesmus, and hydration status were recorded daily. Drug toxicity was monitored with a haematocrit, White blood cell and differential counts, platelet count and urine analysis were performed before, during, and after treatment. The presenting characteristics of the patients were compared using a chi-square statistic. The duration of outcome parameters were examined, using the Kolmogorov-Smirnov Goodness of Fit Test.

RESULTS

One hundred and eighteen patients with age ranging from 6 months-to-65 years were studied and randomized to either the ampicillin (55) or the trimethoprim-sulfamethoxazole (63) treatment groups. Patients in the two groups were comparable with respect to age, sex, presenting complaints, stool exam., and the shigella strains isolated (Table 1). On admission, the percent of patients presenting with fever of 101°F or more (30% vs 28%), severe dehydration (14% vs 15%), or the mean initial WBC (16,000 vs 15,000) did not differ significantly between groups.

The outcome of the treatment for both the regimens was good. However, patients who took trimethoprim-sulfamethoxazole had a significantly shorter duration of fever (mean 1.3 vs 1.5 days), abdominal pain (mean 2.8 vs 3.6 days) and persisting stool mucous (mean 3.9 vs 4.9) and blood (mean 1.5 vs 2.2 days) than those taking ampicillin (Figure 1). There was no difference in the number of days required for the stool to become culture-negative for *Shigella* (3 days), or cleared of faecal leukocytes (<10/hpf) (6 days). Two patients in the ampicillin group had ampicillin-sensitive *Shigella* in their stool 4 days after treatment began. Another 2-year-old female child in the ampicillin group had clinical dysentery for 14 days, even though her stool had no *Shigella* after day 2. On day 14, she was treated with trimethoprim-sulfamethoxazole, and recovered within 48 hours. The mean WBC count and the percent of bands had returned to normal in both groups 5 days after treatment began.

Most of the 118 *Shigella* isolates tested were sensitive to ampicillin (93%), trimethoprim-sulfamethoxazole (97%), as well as kanamycin (98%), gentamycin (100%), and chloramphenicol (96%), and resistant to streptomycin (91%) and tetracycline (93%) (Table 2). Three patients who received ampicillin and had ampicillin-resistant *Shigellae*, were cleared of the organisms by the third day, but their duration of symptoms was longer. One patient with a trimethoprim-sulfamethoxazole-resistant isolate was

TABLE 1

COMPARISON OF *SHIGELLA* PATIENTS RANDOMIZED TO AMPICILLIN (AMP) VS TRIMETHOPRIM-SULFAMETHOXAZOLE (TMS) TREATMENT GROUP

Comparison	Percent of Total AMP (N=55)	TMS (N=63)	P Value*
Age (years)			
0-4	58	59	
5-14	14	16	NS
15+	28	25	
Sex - % Male	64	65	NS
Presenting Complaints			
abdominal pain	98	100	NS
tenesmus	98	97	NS
blood in stool	89	92	NS
vomiting	57	49	NS
fever	95	97	NS
Stool exam			
WBC ≤10	11	9	
11-25	23	29	NS
26+	66	62	
blood	63	63	NS
mucous	96	97	NS
Shigella strains			
dysenteriae 1	47	47	
flexneri	45	44	NS
Other	8	9	

*Chi-square test

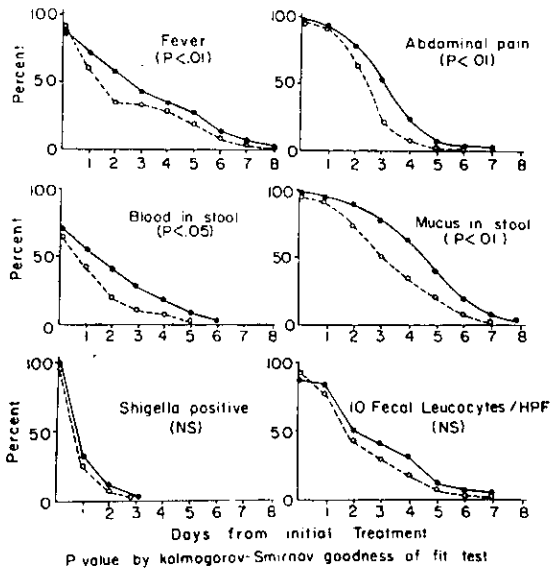

Fig.1: Comparison of Outcome of Treatment of Patients Receiving Ampicillin (•) Vs Trimethoprim-sulfamethoxazole (o) for *Shigella* Dysentery

treated with this drug, and showed good clinical response with clearance of *Shigella* in three days. No clinical or laboratory evidence of drug toxicity was noted, but two patients who received ampicillin and three who received trimethoprim-sulfamethoxazole developed oral thrush.

DISCUSSION

Trimethoprim-sulfamethoxazole is active against a wide range of both gram-positive and gram-negative organisms; and has proved effective in the treatment of urinary tract infections, chronic bronchitis, pneumonia, gonorrhoea, enteric fever, and other infections. *In vitro*, *Shigellae* are highly sensitive to trimethoprim-sulfamethoxazole (11), even in areas where ampicillin-resistant strains have become predominant (12). Earlier

TABLE 2

ANTIBIOTIC RESISTANCE PATTERN OF *SHIGELLA* STRAINS ISOLATED DURING THE TREATMENT TRIAL, MATLAB, 1977-1979

Antibiotics*	% Resistant			
	Shiga (N=55)	Flex (N=53)	Others (N=10)	Total (N=118)
Tetracycline	93	74	75	93
Ampicillin	5	7	20	7
Chloramphenicol	0	8	0	4
Kanamycin	0	5	0	2
Gentamicin	0	0	0	0
Streptomycin	94	89	80	91
Trimethoprim-sulfamethoxazole	5	0	20	3

* Disc Sensitivity

also trimethoprim-sulfamethoxazole has been proved effective in clinical studies for the treatment of shigellosis (13,14). Nelson concluded from a trial similar to our own that trimethoprim-sulfamethoxazole is the best among currently available drugs, in areas where multiple antibiotic resistance is common (8). The present study confirmed these findings in a different geographical location, where the *Shigella* strains are predominantly *flexneri* and *dysenteriae* type 1 and ampicillin resistance has begun to occur.

Several features of the current study should be emphasized. All patients were treated in a field hospital and had clinically severe dysenteric forms of shigellosis (based on our strict entrance criteria requiring dysentery, fever, abdominal pain and tenesmus). Furthermore, nearly 50% of our isolates were *S.dysenteriae* type 1, known to produce more severe dysentery with complications, such as leukomoid reaction and haemolytic-uraemic syndrome (15). Both ampcillin and trimethoprim-sulfamethoxazole were effective in this simple field setting, but patients treated with trimethoprim-sulfamethoxazole had a shorter duration of fever, abdominal pain, tenesmus and stool blood mucous. Almost all patients cleared their stool of *Shigella* within 3 days, including 3 patients in the ampicillin group who had ampicillin-resistant isolates. The prolonged duration of symptoms in these 3 patients suggests that *in vitro* sensitivity testing does correlate with clinical response to treatment, even though other mechanisms were probably important in bacterial clearance.

Recovery from *Shigella* proceeded with daily improvements in different indicators of disease. Clearance of *Shigella* from the stool in the majority of cases occurred in the first day of treatment; and was followed successively by clearance of stool blood (median time one day), faecal leukocytes (median time 2 days) and the return of normal body temperature (median time 2 days). Relief of abdominal pain and clearance of mucous from the stool occurred with a median time of three and four days, respectively. These observations are consistent with our clinical impression that recovery from *Shigella* dysentery can be prolonged due to the process of healing tissue invasion.

In summary, trimethoprim-sulfamethoxazole and ampicillin are both effective in the treatment of severe shigellosis. While candida is a recognized side effect of ampicillin treatment, we found an almost equal number of patients with oral thrush in each treatment group. Few of the *Shigella* isolated in this study were resistant to ampicillin (3) or trimethoprim-sulfamethoxazole (1). Patients treated with trimethoprim-sulfamethoxazole recovered more rapidly, thereby making it the preferred antibiotic in treating severe *Shigella* dysentery. The pattern of antibiotic resistance does seem to correlate with clinical recovery, and should be monitored to ensure the most effective therapy.

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Chapter 18

Changing Pattern of Antibiotic Resistance in *Shigella* Isolated in Bangladesh

M.I. Huq, Q.S. Ahmed and M.M. Rahaman

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

ABSTRACT

Considering the increased evidence of multiple-resistant *Shigellae* in Bangladesh and the consequent problem of treatment, the changing antibiotic sensitivity pattern of *Shigellae* isolated in the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) during the years 1976-1981 was monitored. *Shigellae* were isolated from patients reporting to the hospital and Treatment Centre of the ICDDR,B, Dhaka as well as the camps and Treatment Centre of Teknaf dysentery project. Rectal swabs taken from patients before any therapy started, were plated onto MacConkey and SS agar, and suspected colonies were identified biochemically followed by serotyping with *Shigella* antisera. The antibiotic sensitivity was determined by disc diffusion method using standard Kirby Bauer technique. The 5 antibiotics tested were: tetracycline, ampicillin, chlorampenicol, streptomycin and co-trimoxazole. With each set of plates a control *Escherichia coli* strain (ATCC 25922) was included. The *Shigella* serotype isolated from hospitalized patients was mostly *Shigella flexneri*, followed by *Shigella dysenteriae* type 1. Although there was no appreciable change in the percentage of resistance of *S. flexneri* to tetracycline, all the *S. dysenteriae* type 1 became resistant to tetracycline. Ampicillin resistance in *Shigella* did not show much change. All the different serotypes were commonly resistant to 2 drugs, usually the combination of tetracycline with ampicillin or streptomycin. Only 2 cases each of *S. dysenteriae* type 1 and *S. boydii*, showed resistance to all the five antibiotics. Since *S. dysenteriae* type 1 and *S. flexneri*

account for 75-80% of the total *Shigella* isolated at the ICDDR,B, tetracycline should no longer be considered to be the drug of choice in treating shigellosis. The number of ampicillin-resistant strains of *Shigella* being very small, ampicillin was considered to be the drug of choice. However, it was suggested that antibiotics should be administered after performing proper sensitivity tests.

INTRODUCTION

During the past years, bacillary dysentery due to *Shigella* sp. has been reported from many parts of the world. Hardy and Watt showed *Shigella* to cause most of the acute diarrhoeal diseases in the United States (1). The incidences of *Shigella* have decreased, in USA and other developed countries, mainly due to improved sanitation and health conditions.

As in most developing countries of the world, diarrhoea causes a great deal of morbidity and mortality in Bangladesh. Almost 80% of the bacillary dysentery reported to the ICDDR,B hospital are due to *Shigella* sp. The rest are due to salmonella, Campylobacter, EPEC and other enteroviruses. The percentage of *Shigella* isolation in Dhaka and its suburb increased from 0.6% in 1970 to 14% in 1974 and approximately 18% in 1977. The increase in isolation rate of the *Shigella* also paralleled with the increase in resistance to different commonly used antibiotics in course of time. It was also observed that during this period majority of the isolates acquired resistance to more than one antibiotic.

Shigella strains resistant to multiple antibiotics were first recognized in 1955. Since that time, epidemics of shigellosis due to multiple-resistant organisms have been documented in Japan (2), England (3) and Central America (4). In Japan during 1968-1970, about 93% of *Shigella* isolates were resistant to one or more of the drugs streptomycine, tetracycline, chloramphenicol and sulphadiazine. Our observations on the *Shigella* isolates in Dhaka showed that by 1970, 75% of *Shigella dysenteriae* type 1 and 27% of *Shigella flexneri* were multiple-resistant, which increased to approximately 96% and 68% by 1975 (5). This phenomenon of resistance to one or more antibiotics has created a serious problem for the clinician in choosing the proper antibiotic for the treatment of acute shigellosis.

Considering the increased evidence of multiple resistant *Shigellae* in Bangladesh and the consequent problem of treatment, we began monitoring the changing antibiotic sensitivity pattern of *Shigellae* isolated in the ICDDR,B during the past years.

MATERIALS AND METHODS

The *Shigella* included in this study were isolated from patients reporting to the hospital and Treatment Centre of the ICDDR,B Dhaka as well as to the camps and Treatment Centre of Teknaf dysentery project. Rectal swabs were taken from patients before any therapy started, and plated onto MacConkey (6) and SS Agar (7). Suspected non-lactose fermenting colonies were picked from either of the above plates and identified biochemically, followed by serotyping with commercially available *Shigella* antisera. The sensitivity of each of the isolates to different antibiotics was determined by disc diffusion method using standard Kirby Bauer technique (8). The commercially available standard disc (BBL) containing tetracycline (30 mcg), ampicillin (10 mcg), chloramphenicol (30 mcg), streptomycin (10 mcg) and trimethoprim-sulfamethoxazole (1.25 mcg - 23.75 mcg),were used routinely. Each *Shigella* strain tested was inoculated into T_1N_1 (Trypticase 1% and Sodium Chloride 1%) broth, and a growth density comparable to standard Barium sulphate density was used as the inoculum on the Muller Hinton Agar plate. The antibiotic discs were placed on the lawn and incubated overnight. With each set of plates a control *E.coli* strain (ATCC 25922) was included. The clear zone of lysis around the disc was measured and interpreted as sensitive or resistant.

The presence of R. factor in the resistant strains was tested *in vitro* by conjugation with standard *E.coli* recipient strain $K_{12}F$ lac^+,which is resistant to nalidixic acid but sensitive to all antibiotics (9). Equal volumes of 6 hour broth cultures of resistant donor (*Shigella* sp.) and recipient *E.coli* were mixed in a test tube in presence of broth and incubated for 18 hours at 37°C. One measured loopful of the incubated mixture was streaked onto special MacConkey plate containing no bile salt but with nalidixic acid and one of the antibiotic on test. After incubation for 18-24 hours, resistant enconjugants, if any, were picked out. Transfer frequency is expressed as the proportion of resistant progeny per recipient cells.

RESULTS

Table 1 shows the yearly isolation rate of the different serotypes of *Shigella* at the ICDDR,B hospital during the years 1976-1981. The *Shigella* serotypes isolated from hospitalized patients were mostly *Shigella flexneri*, which is followed by *Shigella dysenteriae* type 1 *(Shigella shiga)*. Though isolation rates varied from year-to-year during this period, there was no significant change in the overall isolation rate of the different *Shigella* serotypes.

Table 2 shows the resistance pattern of different *Shigella* serotypes to various antimicrobial agents. It is worth mentioning that though there was no appreciable change in the percentage of resistance of *Shigella flexneri*

TABLE 1

NUMBER AND PERCENTAGE OF *SHIGELLA* ISOLATED AT ICDDR,B
DHAKA HOSPITAL DURING JANUARY 1976 TO MAY 1981

Year	*S. shiga* No.	%	*S. dysenteriae* type (2-10) No.	%	*S. flexneri* No.	%	*S. boydii* No.	%	*S. sonnei* No.	%	Total *Shigella*
1976	1134	23	221	2	2987	67	266	5	251	5	4859
1977	353	10	122	4	2509	72	252	7	229	7	3465
1978	73	10	28	4	555	72	50	7	62	8	768
1979	141	19	17	2	545	72	41	5	12	2	755
1980	182	16	50	4	753	67	42	4	30	8	1117
May 1981	15	5	18	6	254	80	24	8	7	2	318

TABLE 2

ANTIBIOTIC RESISTANCE OF *SHIGELLA* STRAINS
ISOLATED BETWEEN JANUARY 1978 - DECEMBER 1980

		No. and % of All Isolates Resistant									
		Te		S		Am		C		Sxt	
Year	Strain	No.	%	No.	%	No.	%	No.	%	No.	%
1978	*S. shiga*	52	93	51	91	3	5	5	8	0	0
	Dysent. 2-10	4	15	18	69	0	0	0	0	0	0
	S. flex.	336	78	393	91	24	6	11	3	0	0
	S. boydii	26	59	43	98	10	23	1	2	0	0
	S. sonnei	17	29	45	78	8	14	3	5	0	0
1979	*S. shiga*	130	94	100	72	4	3	6	4	0	0
	Dysent. 2-10	3	12	18	72	1	4	0	0	0	0
	S. flex.	666	81	578	70	77	9	44	5	0	0
	S. boydii	36	65	43	78	3	5	0	0	1	2
	S. sonnei	13	43	7	23	3	10	0	0	0	0
1980	*S. shiga*	136	100	127	93	10	7	2	1	0	0
	Dysent. 2-10	11	25	20	45	4	9	2	5	0	0
	S. flex.	673	85	644	88	58	7	34	4	0	0
	S. boydii	27	68	31	70	1	3	1	3	0	0
	S. sonnei	2	33	4	67	2	33	0	0	0	0

to tetracycline during these years, all the *Shigella shiga* became resistant to tetracycline. Ampicillin resistance in *Shigella* did not show much change excepting *Shigella sonnei*, which showed increased percentage of resistance. *Shigella* serotypes resistant to multiple antibiotics are shown in Table 3. It has been observed that resistance to 2 drugs was most common with all the different serotypes with usual combination of tetracycline with ampicillin or streptomycin. Only in two cases each of *Shigella shiga* and *S.boydii* resistance to five antibiotics (tetracycline, ampicillin, chloramphenicol, kanamycin, streptomycin or septrin) was developed.

TABLE 3

MULTIPLE RESISTANT PATTERN OF *SHIGELLA*
ISOLATED DURING JANUARY 1978 - DECEMBER 1980

Year	Strain	Total isolation tested	Percentage Resistant To					
			0 drug	1 drug	2 drug	3 drug	4 drug	5 drug
	S.shiga	55	0	14	79	5	0	2
	Dysent. 2-10	26	23	62	8	4	4	0
1978	*S.flex.*	430	4	23	68	2	3	0
	S.boydii	48	10	21	63	6	0	0
	S.sonnei	58	16	59	16	10	0	0
	S.shiga	138	4	24	67	5	0	0
	Dysent. 2-10	25	28	60	8	0	4	0
1979	*S.Flex*	833	9	27	55	4	5	0
	S.boydii	55	13	27	56	2	0	2
	S.sonnei	30	27	30	27	13	3	0
	S.shiga	136	1	7	86	4	3	0
	Dysent. 2-10	44	52	28	14	6	0	0
1980	*S.flex.*	788	9	7	76	4	4	1
	S.boydii	40	22	12	65	0	0	0
	S.sonnei	89	55	13	23	5	2	0

* 0 drug = Non antibiotic-resistant, 1 drug = Te or S: 2 drug = Te, S or Am: 3 drug = Te, Am, S or C, 4 drug = Te, Am, K, S, or C
5 drug = Te, Am, C, K, S or Sxt.

DISCUSSION

Drug resistance in shigellosis has been a great concern to physicians. In case of bacillary dysentery the clinician usually starts on symptomatic treatment, paying little attention to fluid loss and electrolyte balance. The antibiotic plays a significant role in decreasing the duration and volume of diarrhoea. In Bangladesh, usually in most cases, the antibiotic is administered without looking for the bacteriological findings or the antibiogram of the pathogen causing the disease. With the emergence of multiple antibiotic-resistant organisms, treatment has become more of a problem. The resistance pattern of multiple-resistant strains of *Shigella* appears to be related to the type of antibiotics to which they were exposed. In Japan, it's mostly resistant to tetracycline, chloramphenicol, streptomycin and sulphonamide; in Central America to ampicillin, chloramphenicol and tetracycline. The evergrowing pattern seen in Bangladesh is resistance to streptomycin and tetracycline with chloramphenicol and/or ampcillin. In one of our studies in a Baby Home in Dhaka we found that *Shigella* strains isolated from the cases were resistant to tetracycline, ampicillin, chloramphenicol, kanamycin, streptomycin and trimethoprim-sulfamethoxazole, and sensitive to gentamicin only (10). In each of the above areas, the resistance pattern reflects the heavy and often indiscriminate use of antibiotics.

While studying the antibiotic sensitivity pattern of the *Shigella* isolates from Teknaf, we found a striking increase in the resistance pattern during the two years of our study. In 1977, we observed the influx of refugees from Burma, resulting in the increase of cases due to *Shigella;* and for the first time we started getting sulphonamide-sensitive *Shigella* (in the refugee camps). During this time, resistance to tetracycline was less, but this increased in 1978. This acquisition of resistance by a population, which had had little or no access to antibiotics in the Camps, is very interesting. The development of transferable resistance to multiple antibacterial drugs in enteric bacteria is of critical importance. In our study, we have found that all the strains of multiple-resistant *Shigella* could transfer the full spectrum of resistance present in the donor to the recipient *E.coli* strains; However, streptomycin resistance could be transferred from only 6/14 strains. The *E.coli* isolated from these patients was also found to have acquired multiple antibiotic resistance, which may have contributed to its transfer of resistance factor to sensitive *Shigella* strains. It has been observed that dysentery due to some *Shigella* species is often associated with complicated clinical syndromes (11). These include granulocytic leukemoid reaction, with a fall in haematocrit associated with striking erythrocyte fragmentation and transient renal failure. Prompt treatment with an appropriate antibiotic might prevent these side effects and decrease the duration of diarrhoea.

Since *Shigella dysenteriae* type 1 and *Shigella flexneri* account for about 75-80% of the total *Shigella* isolates at the ICDDR,B, tetracycline should no longer be considered to be the drug of choice in treating

shigellosis. Although we have isolated a few ampicillin-resistant strains of *Shigella*, the number is very small compared to that in other countries. Ampicillin should be considered the drug of choice for the treatment of shigellosis. However, it is advisable to administer antibiotics after proper sensitivity testing on the pathogen is done.

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Chapter 19

Epidemiologic and Clinical Features of Patients with *Shigella* Attending a Diarrhoeal Disease Hospital in Bangladesh

Barbara J. Stoll[1], Roger I. Glass[2], M. Imdadul Huq[1], M.U. Khan[1], Hasina Banu[1], James Holt[3]

[1]*International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh,*
[2]*On assignment from the Centers for Disease Control, Atlanta, Georgia, U.S.A.*
[3]*Department of Biostatistics, Mount Sinai School of Medicine, New York, U.S.A.*

ABSTRACT

We have reviewed the epidemiologic and clinical characteristics of 412 patients with *Shigella*, from a 4% systematic sample of approximately 100,000 patients attending the Dacca Hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh between December 1, 1979 and November 30, 1980. *Shigella* was isolated from 11.6 percent of the 3550 patients in the sample, and was the second most common isolate in patients over 2 years of age.

We found two presentations of *Shigella:* watery diarrhoea occurring in younger children and associated with a shorter duration of illness, more vomiting and more dehydration; and dysentery associated with stool blood and abdominal pain. These different presentations may reflect two different mechanisms in the pathogenesis of this disease: toxin-mediated versus tissue-invasive disease. Simple visual inspection of stool for blood correctly identified 44% of all *Shigella* patients, and could be one simple criteria for initiating therapy in the field.

The current international emphasis in diarrhoeal disease therapy focuses on oral rehydration fluid, and may be less effective in areas having a high prevalence of *Shigella* and other primarily non-dehydrating diarrhoeas. *Shigella* is a common and severe diarrhoeal illness in Bangladesh warranting more clinical and public health attention.

INTRODUCTION

In October 1979, a surveillance system was set up at the Dacca Hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) to study the epidemiologic, clinical and laboratory characteristics of the approximately 100,000 patients who come to the hospital for care each year. This paper outlines the methods used for surveillance and reviews our data on *Shigella* patients seen between December 1, 1979 and November 30, 1980. We have looked at the distribution of *Shigella* patients by age, sex and season; have examined their presenting signs, symptoms and hospital course, and have reviewed the patterns of antibiotic resistance of *Shigella* isolates. Shigella is an important cause of severe diarrhoeal disease in Dacca and the second most common enteric pathogen in patients over 2 years.

MATERIALS AND METHODS

Background.

The ICDDR,B, formerly the Cholera Research Laboratory, has operated a hospital in Dacca, the capital and largest city of Bangladesh, since 1962, which provides care free of charge to patients with diarrhoeal disease. The majority of patients are treated in an outpatient area. Patients with more severe illness are admitted either to an intravenous treatment centre or to a small hospital ward.

In recent years, as the number of patients increased, it has become difficult to collect useful information on all patients. Therefore, a surveillance system was set up to study a representative sample of these patients. Between October 1979 and February 1980, a 2% sample of patients was selected, but the sample was increased to 4% in March 1980 for statistical reasons. Surveillance patients are seen by the regular hospital staff, with emergency cases treated on a priority basis. After initial examination and care by a nurse or physician, the patient or an adult guardian is interviewed by a special health assistant who collects uniform information on demographic factors, recent medical history, presenting symptoms, previous therapy and therapy prescribed at ICDDR,B. A physical examination, including careful assessment of dehydration, is performed by a physician. Each patient is weighed and measured at discharge. A rectal swab for culture is obtained from all patients and a stool sample for microscopic examination is requested. If a patient is admitted to the intravenous treatment centre or hospital ward, information on hospital course is collected as well.

In Bengali, the word for dysentery is "amasha". The definition of "amasha" is imprecise; it may mean stool with mucus, stool with blood or both. Throughout the study period we asked patients if they had stool mucus or stool blood. From February 1980, we have asked specifically about dysentery (amasha) and watery diarrhoea (N=367 of 412 shigella patients).

The percentage of the Harvard reference weight-for-height (1) was determined for all children ≤8 years, to assess nutritional status.

Laboratory Methods.

Rectal swabs or stool samples were plated directly on salmonella-shigella (SS), taurocholate-tellurite-gelatin (TTGA), MacConkey's and Campy-BAP[2] (BBL Microbiology Systems) agars. Specimens were also enriched in alkaline peptone water for *Vibrios* and then plated on TTGA. The plates were examined for *Salmonella, Shigella, Vibrios* and *Campylobacter jejuni* by standard methods (2,3,4). Non-lactose fermenting colonies from MacConkey's and SS agar were further screened on Kligler's iron agar slants and motility indole urea media. *Shigella* isolates were biochemically identified and serologically grouped with a slide agglutination test using commercially available antisera (Burroughs Wellcome). If the test was negative, a saline suspension was prepared and heated for 30 minutes at 100°C to remove heat-labile surface antigen, and the slide agglutination test was repeated.

Throughout the study period, *Shigella* isolates were tested for sensitivity to tetracycline, ampicillin, chloramphenicol, kanamycin and gentamicin, using the Bauer-Kirby method (5). Four-to-five-hour broth cultures of *Shigella* isolates were plated on Mueller-Hinton agar, commercial antibiotic discs (Baltimore Biological Company) were applied, and the size of a zone of inhibition was measured after 24 hours.

A pool of 10 colonies of *E.coli* from each patient was tested for the production of heat-labile toxin (LT) in the Chinese hamster ovary cell assay, and for heat-stable toxin (ST) in the infant mouse assay (6). Rotavirus antigen was identified by enzyme-linked immunosorbent assay (7). Stool samples were also examined for red blood cells, white blood cells and parasites, by direct smear without concentration techniques.

Results were computerized and analyzed using chi-square statistics.

RESULTS

Between December 1, 1979 and November 30, 1980, 3550 patients were studied -- a representative sample of the approximately 100,000 patients who came to ICDDR,B during that year. Twenty-five percent of patients were less than 1 year of age, 37% were between 1 and 5 years and 39% were 5 years and over. Sixty percent of patients seen were male.

Isolation Rates.

Shigella and *Campylobacter jejuni* were isolated with equal frequency from 11.6% of all patients. Enterotoxigenic *E.coli* (ETEC) and rotavirus were found in 20% and 19% of patients, respectively Since rotavirus and

Campylobacter jejuni occurred most frequently in children under 2 years of age, an age group comprising 51% of our population, *Shigella* was the second most common isolate in patients over 2 years. Of the 412 *Shigella* isolates, 270 (66%) were *Sh.flexneri*, 66 (16%) *Sh.dysenteriae* type I, 31 (7%) *Sh.sonnei*, 24 (6%) *Sh.dysenteriae* type II and 21 (5%) *Sh.boydii*.

Shigella was isolated most frequently in the elderly (60 years +), least frequently in children under 1, and with similar frequencies in the other age groups, with no major differences in the age distribution by species (Table 1). There was no significant difference in the sex distribution of *Shigella* patients in any age group.

TABLE 1

AGE DISTRIBUTION OF PATIENTS WITH *SHIGELLA*
DACCA HOSPITAL SURVEILLANCE
DECEMBER 1979 - NOVEMBER 1980

Age (Years)	Patients Studied N	*Shigella* Species (Rate/1000)					All *Shigellae*
		Flex	Dys I	Sonn	Dys II	Boyd	
<1	876	41	8	6	6	2	65
1-2	943	99	17	13	10	5	143
3-4	367	74	27	11	8	3	123
5-9	316	60	38	16	6	10	130
10-14	122	90	16	8	0	16	131
15-29	459	85	33	4	2	9	133
30-44	296	88	10	0	7	10	115
45-59	114	70	0	18	9	9	105
60+	57	193	18	0	18	0	228
All ages	3550	76	19	9	7	6	116

Seasonality.

Shigella was isolated most frequently in the winter (October - January) and in the hot summer (April - May), a pattern reflecting the seasonality of the most common isolate, *Sh.flexneri* (Figure 1). The winter peak corresponded to the time of the year with the lowest mean temperature and rainfall. The smaller summer peak occurred during the hot dry period, just before the monsoon rains began. The lowest isolation rate, in February, corresponded to the coldest month of the year. The trough in August occurred during

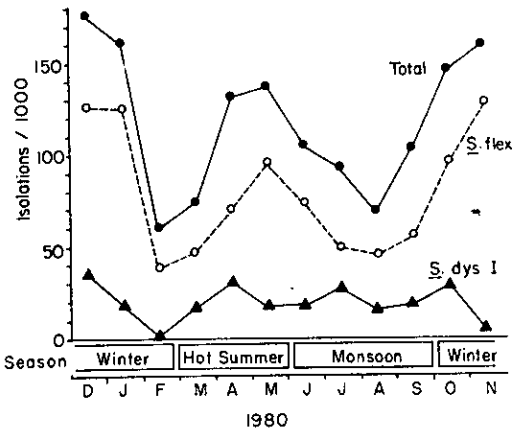

Fig. 1: Seasonal distribution of patients with *Shigella* attending the ICDDR,B hospital, Dhaka, December 1979-November 1980.

the monsoon. This pattern was the same for all species, although the numbers of isolates of species other than *Sh. flexneri* and *Sh.dysenteriae* type I were small.

Clinical Characteristics - Treatment - Outcome.

Patients with *Shigella* presented with both dysentery and watery diarrhoea, but complained more of bloody mucoid stools and abdominal pain and less of watery diarrhoea and vomiting than patients without *Shigella* (Table 2) Among the different *Shigella* species, patients with *Sh.dysenteriae* type I had more dysentery with stool blood and abdominal pain and patients with *Sh.sonnei* had more watery diarrhoea and vomiting.

The duration of illness ranged from less than one day to over 3 months. Forty-nine percent of patients presented with an acute illness of 0-3 days, 23% had been ill for 4-6 days, 17% for 7-13 days and only 11% for 2 weeks or longer . Patients with *Shigella* had less moderate or severe dehydration and were treated with less intravenous fluid and more medications, primarily antibiotics, than patients without *Shigella*. Eighteen surveillance patients died in the hospital (case fatality rate, 0.5) and three of these had *Shigella* (case fatality rate, 0.7).

Poor nutritional status and failure to breastfeed have been considered to be risk factors for shigellosis in children (8), so we examined these associations in our population. There was no significant difference in the nutritional status (i.e., weight-for-height) between children (≤ 8 years) with *Shigella* and those without, but fewer children aged 0-2 years with *Shigella* were breastfed (59% vs. 78% $p<.05$). We compared children (0-2 years) with *Shigella* who were breastfed (N=92) with those who were not (N=63), and found that the breastfed group had less severe illness: i.e., fewer required intravenous therapy (16% vs. 38%, $p<.01$), fewer were admitted to the inpatient ward (5% vs. 19%, $p<.01$) and fewer were significantly malnourished (34% vs. 48% less than 80% weight-for-height, $p<.01$).

To examine possible mechanisms of the disease, we compared *Shigella* patients who complained of watery diarrhoea only with those who complained of dysentery only (Table 3). Patients with watery diarrhoea had a shorter duration of illness, complained of less stool blood and abdominal pain but more vomiting, were more severely dehydrated, and were more often treated with intravenous fluid in the intravenous centre and ward. These results were the same when patients having other enteric pathogens in addition to *Shigella* were excluded.

TABLE 2

CLINICAL CHARACTERISTICS-TREATMENT-OUTCOME PATIENTS WITH *SHIGELLA*
DACCA HOSPITAL SURVEILLANCE
DECEMBER 1979 - NOVEMBER 1980

	Flex N=270 %	Dys I N=66 %	Sonn N=31 %	Dys II N=24 %	Boyd N=21 %	All Shigella N=412 %	All Non-shigella N=3138 %
Symptom							
stool mucus	88	97	55	83	76	86	56
stool blood	55	83	10	41	48	55	15
abdominal pain	68	85	52	63	71	70	54
watery diarrhoea	33	22	74	41	52	30	69
vomiting	32	41	61	46	43	37	61
Duration (days)							
0-3	49	42	53	54	67	49	60
4-6	20	36	10	25	24	23	16
7-13	17	17	30	8	9	17	12
14+	14	5	7	13	0	11	12
Physical exam							
Temperature							
$\leq 100^5$	94	91	97	92	90	93	97
$> 100^5$	6	9	3	8	10	7	3
Dehydration							
none	53	50	42	46	48	51	41
mild	34	25	39	46	43	36	38
moderate	11	15	16	8	10	12	19
severe	1	0	3	0	0	1	2
Treatment							
oral fluid	61	70	71	67	62	64	60
IV fluid	24	29	32	8	24	24	34
medication	87	98	73	88	67	87	61

Comparisons between all *Shigella* and non-*Shigella* patients were significant ($p<.01$) for all variables except oral fluid

TABLE 3

WATERY DIARRHOEA VS DYSENTERY PRESENTATIONS AMONG PATIENTS WITH *SHIGELLA* AS ONLY ENTERIC PATHOGEN
DACCA HOSPITAL SURVEILLANCE
FEBRUARY 1980 - NOVEMBER 1980

	PRESENTATION		
	Watery Diarrhoea N=66 %	Dysentery N=159 %	P. Value
Symptom			
stool blood	2	83	< .01
stool mucus	52	17	< .01
abdominal pain	62	84	< .01
vomiting	55	25	< .01
Duration (days)			
0-1	39	11	
2-3	24	35	
4-6	12	29	< .01
7-13	15	14	
14+	8	11	
unknown	2	-	
Dehydration			
non-mild	82	92	< .05
moderate-severe	18	8	
Treatment			
oral fluid	71	73	NS
IV fluid	35	14	< .01
medication	67	91	< .01

To determine if there was a difference in disease pattern between infants, children and adults, we looked at *Shigella* patients in different age groups. There was no difference in duration of illness or in most presenting signs and symptoms by age. Patients over 5 more commonly complained of abdominal pain (90% vs 56%, p<.001) which may represent underreporting of abdominal pain by mothers of small children. Young children under 3 years and patients over 45 years were more likely to have severe illness requiring hospitalization in the inpatient ward (11% vs 4% in other age groups, p<.01). Two of the three *Shigella* deaths occurred in patients less than 1 year.

Stool Examination.

Microscopic examinations were performed on stool specimens from 74% of *Shigella* patients (Table 4). Blood on visual examination of stool correlated closely with the presence of 10 or more red blood cells per high power field. It was a good discriminator of *Shigella* and was found in 44% of patients with *Shigella*, but only 8% of non-*Shigella* patients. Stool mucous was found in 85% of all patients, and was not helpful in the diagnosis of *Shigella*. *Shigella* stools had significantly more white blood cells and were more likely to have an alkaline pH.

TABLE 4

STOOL EXAMINATION OF PATIENTS WITH *SHIGELLA*
DACCA HOSPITAL SURVEILLANCE
DECEMBER 1979 - NOVEMBER 1980

Examination	Flex N=196 %	Dys I N=56 %	Sonn N=20 %	Dys II N=18 %	Boyd N=14 %	All Shigella N=304 %	All Non-shigella N=1930 %
Visual:							
blood	39	82	21	19	25	44	8
mucous	93	100	84	88	100	94	84
Microscopic:							
RBC							
0	24	11	50	33	36	25	68
1-10	35	11	35	56	36	32	24
>10	40	79	15	11	29	43	7
WBC							
0	2	0	0	0	0	1	4
1-10	18	5	50	22	36	19	58
>10	80	95	50	78	64	80	38
pH:							
alkaline	64	92	68	60	33	68	43
acid	36	8	32	40	67	32	57

Comparisons between all *Shigella* and non-*Shigella* patients were highly significant ($p<.01$) for all variables.

Antibiotic Sensitivity.

Antibiotic sensitivity testing was performed on 396 of the 412 *Shigella* isolates (96%). All isolates were sensitive to gentamicin; there was little resistance to ampicillin (3%), chloramphenicol (2%) and kanamycin (1%); but

seventy-eight percent were resistant to tetracycline. Tetracycline resistance ranged from 100% in *Sh.dysenteriae* type I to 18% in *Sh.dysenteriae* type II. Twelve strains (3%) had a pattern of multiple antibiotic resistance, and in seven of these, resistance was to 3 antibiotics (tetracycline, ampicillin, chloramphenicol =4, tetracycline, ampicillin, kanamycin =3) (Table 5).

TABLE 5

ANTIBIOTIC RESISTANCE PATTERN OF *SHIGELLA* ISOLATES
DACCA HOSPITAL SURVEILLANCE
DECEMBER 1979 - NOVEMBER 1980

	Percent Resistant					
Antibiotic	Flex N=263	Dys I N=62	Sonn N=30	Dys II N=22	Boyd N=19	Total N=396
Tetracycline	83	100	37	18	79	78
Ampicillin	3	2	3	0	0	3
Chloramphenicol	2	2	0	0	0	2
Kanamycin	<1	2	3	0	0	1
Gentamicin	0	0	0	0	0	0

DISCUSSION

The importance of *Shigella* as an enteric pathogen in Bangladesh has only been appreciated in recent years (9,10,11). In Dacca Hospital, 11.6% of all patients or approximately 12,000 patients per year have *Shigella*. It is the second most common isolate in patients over 2 years and the third most common isolate in all patients. As in other developing countries (12,13,14,15), *Sh.flexneri* and *Sh.dysenteriae* type I predominate. Isolation rates found in Dacca are twice those found in the riverine area of Matlab, Bangladesh (16), suggesting a possible urban/rural difference.

Our lowest isolation rates were in children under 1. The rise in *Shigella* isolation rates after 1 year of age suggests that breast milk is protective or that children less than 1, being less mobile, have less exposure. The fact that fewer children with *Shigella* were breastfed and that those who were had a less severe illness supports a possible protective role for breast milk.

Shigella strains were found to be highly resistant to tetracycline. This finding has been reported recently for *Shigella flexneri* in Dacca (17), and may be related to the overuse of tetracycline. Multiple antibiotic resistance, found in twelve strains, might be the result of an R-factor coded drug resistance.

Two mechanisms are presently implicated in the pathogenesis of shigellosis: toxin production affecting the small bowel, and resulting in watery diarrhoea, and tissue invasion of the colon, resulting in classical bacillary dysentery (18,19,20,21,22). Our data support these two mechanisms. In our population, patients with *Shigella* presented with both watery diarrhoea and dysentery and their clinical pictures were distinct from one another. *Shigella* patients with watery diarrhoea had a shorter duration of illness, more vomiting, less abdominal pain and were more severely dehydrated. Their illnesses were similar to other watery diarrhoeas, such as those caused by enterotoxigenic *E.coli*, and were consistent with the action of an enterotoxin.

International diarrhoeal disease control programmes have concentrated on the use of oral rehydration fluid, which is effective in watery, dehydrating diarrhoeas. Dysenteries of various aetiologies are less amenable to simple oral rehydration therapy, and may require antibiotics, antiamebics or other medications. The precise aetiology is more important in deciding the appropriate therapy for dysentery than for watery diarrhoea. There is a great need for simple techniques to diagnose bacillary and other dysenteries in a field setting which can be used by minimally-trained health workers. We are currently developing models for simple diagnosis. From this preliminary data, the most useful symptoms for *Shigella* diagnosis were the complaints of stool blood and abdominal pain and the absence of watery diarrhoea and vomiting in patients over age 1. Visual inspection of stool proved to be most discriminating. The presence of visible blood, an observation that can be made by anyone, correctly identified 44% of *Shigella* patients. Moreover, if all patients with visible blood had been treated for *Shigella*, we would have treated those 44% appropriately.

In conclusion, *Shigella* is a common and severe diarrhoeal disease in Dacca, warranting more concerted clinical and public health attention. The Dacca Hospital surveillance system is a resource from which future studies on *Shigella* can be planned.

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Discussions

Dr. A.M. Molla

I have some information on diarrhoea due to *Shigella* in children, that I would like to add to the wealth of information presented this morning. At the ICDDR,B we have studied food intake and absorption of nutrients during diarrhoea due to different aetiologies in children. A balance study of 72 hours, using charcoal as a marker, was conducted during acute diarrhoea and 2 and 8 weeks after recovery. We originally planned to study cholera, *E.coli* and rotavirus patients. But a group of 8 patients clinically indistinguishable from acute watery diarrhoea due to other aetiologies, but later confirmed to be *Shigella* infection, was included. I will present to you the food intake and absorption of carbohydrates, fats, calories and proteins in these *Shigella* cases; and compare them with those of cholera, rotavirus and ETEC diarrhoea. Calorie intake during acute diarrhoea due to different aetiologies was reduced by 30-40%. The intake of calories-per-day in the acute stage was 74.8 K.cal/kg in cholera, 68.5 K.cal/kg in rotavirus, and 70.6 K.cal/kg in *Shigella*. In cholera, *E.coli* and *Shigella*, the intake improved, and reached 115 to 116 K.cal/kg/day. However, rotavirus patients took longer (2 weeks) to reach the same level.

During the acute stage, absorption of carbohydrates was 78.6% in cholera, 86.7% in *E.coli*, 68.1% in rotavirus and 56% in *Shigella*. Carbohydrate absorption improved further, to 80-90% within 2 weeks after recovery, and remained at that level at 8 weeks after recovery. Protein and fat absorption was about 50% during the acute stage, and improved during the recovery stage, except in *E.coli* and *Shigella* diarrhoea, in which cases protein malabsorption continued up to 12 weeks. The cause of protein malabsorption in *Shigella* is not known. There are two possibilities: that in *Shigella* infection, the exudative loss of protein continues longer; or that the small intestine also is involved in causing diarrhoea, perhaps toxin-mediated diarrhoea. It must be made clear that we are talking about *Shigella*-confirmed patients, clinically indistinguishable from cholera patients. Severe invasive *Shigella* dysentery could well be different with respect to absorption. But we have not studied this.

Chapter 20

Host Defenses to *Shigella*

William P. Reed

Veterans Administration Hospital, Albuquerque, New Mexico, U.S.A.

ABSTRACT

When *Shigella* are ingested by non-immune individuals, the bacteria are capable of adhering to the intestinal wall and entering through intestinal epithelial cells into tissue beneath the epithelium. Once in this location the *Shigella* encounter highly effective host defense mechanisms which kill them. If organisms are susceptible to serum alone, minute quantities of IgM, and greater quantities of IgG, present even in non-immune individuals, will promote killing by complement, through either the classical or alternative complement pathways. Serum-resistant organisms are opsonized by IgG or by IgM-plus complement, and are killed by phagocytic cells. Perhaps the reason that bacteremia is not commonly seen in healthy well-nourished individuals with shigellosis is because these killing mechanisms are so effective, even in persons who are non-immune.

When *Shigella* are ingested by immune individuals who have anti-*Shigella* secretory IgA in the intestinal tract, these antibodies inhibit attachment of *Shigella* to the intestinal epithelium, and thus prevent penetration. They may also inhibit absorption of toxin.

Other bacterial flora may also assist the host in resisting shigellosis, either by their inhibitory effect on growth of *Shigella*, or by inducing host resistance, which may be through cross reacting antigens.

BACTERIAL ADHERENCE, FIRST STEP IN PATHOGENESIS OF MANY INFECTIONS

We live surrounded by a sea of bacteria, and our health depends in no small measure on an ability to keep them from invading our tissues. Growth and spread of bacteria are promoted by numerous offensive devices which we counter with a variety of host defenses, including the ability of skin and mucous membranes to resist penetration by microorganisms. One feature that may aid bacteria in tissue invasion is an ability to adhere to epithelial surfaces. This ability enables microorganisms to colonize skin and mucous membranes, and in case of cutaneous injury large numbers may be available to penetrate directly into deeper tissues. A few organisms, such as gonococci, *Shigellae* and *Salmonellae*, may be capable of breaching even normal mucosa, but logically as well as experimentally the first step in pathogenesis of these diseases appears to be attachment of the bacteria to epithelial cells (1-2). There is some evidence available to support the concept that bacterial adherence may also be the first step in the pathogenesis of numerous other infections, including pharyngitis, urinary tract infection, bacterial endocarditis, infection of prothetic devices and perhaps even dental caries. Bacteria may attach through several different mechanisms, as shown in Table 1.

TABLE 1

Organisms	Type of infection	- Currently recognized - mechanism of adherence
Streptococcus mutans	Dental caries	Dextran containing polysaccharide manufactured from sucrose
Staphylococcus epidermidis	Ventriculo-atrial shunt infections	Mucoid substance elaborated by organism
Group A *Streptococcus pyogenes*	Pharyngitis	Lipoteichoic acid in fimbriae
Enteric organisms	Cystitis and pyelonephritis	Pili
Intestinal organisms (*E.coli, Shigella, Salmonella, Vibrio,* other bacteria)	Diarrhoea or no disease	Fimbriae, flagellae. Pili
Neisserio gonorrhea	Urethritis	Pili
Enterococcus and *Streptococcus viridans*	Endocarditis	Unknown

An early indication that attachment of bacteria to epithelial surfaces was important in the pathogenesis of enteric infections came from the

observation of LaBrec, Formal and co-workers that *Shigella* must penetrate into the mucosa before they cause disease (2). Subsequent studies on immunity to *Vibrio cholera* by Fubara and Freter gave additional early clues that the association of bacteria with epithelium is important in the pathogenesis of toxin-induced disease, even in the absence of penetration (3). In the case of cholera, it may be that the association with the mucosa prevents the rapid flow of intestinal fluids from washing the organisms away, or it may be that toxin production near the epithelial surface leads to more effective absorption. Some organisms, such as enteropathogenic *E.coli*, cause diarrhoea, especially when they proliferate in the small intestine, a region which is normally relatively free from bacteria (4). The ability to adhere to small bowel mucosa may be one of the features that is necessary for such organisms to cause disease, and one of our immune mechanisms may be an ability to prevent this attachment (5). Fimbriae, flagellae, and perhaps pili may each serve as the organelle for attaching different bacteria to the intestine (6).

The mechanisms by which *Shigella* attach to colonic mucosa has not been clarified, although adherence has been shown to be inhibited by the presence of mannose (6,7). Also it has been shown that colonic contents from conventional or *Shigella* monocontaminated mice, but not from germ-free mice, modify the epithelial surface to promote *Shigella* attachment (8). Presumably, bacterial enzymes, including glycosidases, may modify surface components on the epithelial cells to make them receptive to attachment by *Shigella*.

PENETRATION OF MUCOSA BY *SHIGELLA*

LaBrec and co-workers showed that *Shigella* were capable of epithelial cell penetration, and postulated that such penetration was an essential step in the pathogenesis of shigellosis (2). Subsequently, it has been shown that some *Shigella* produce toxins that might cause diarrhoea, but still epithelial cell penetration seems to be very important in the genesis of the disease. Recently, Hale and coworkers have shown that penetration into tissue culture cells requires active metabolic participation from both the bacteria and the cell that it enters (9,10).

Although virulent *Shigella* readily penetrate intestinal epithelium, the penetration is frequently only superficial.

ANTIBODY RESPONSE

The epithelial surface that separates the lymphoid aggregates referred to as Peyer's patches from the intestinal lumen, consists of specialized flattened epithelial cells called "M" cells (11). These cells are capable of taking up intraluminal antigen, which is present in such low concentrations that ordinary epithelial cells do not take up detectable quantities. The

antigens traverse these cells and are released into the interstitial spaces of Peyer's patches, where they encounter lymphoblastoid B lymphocytes. These B cells operate under the influence of both helper and suppressor T cells to produce antibody, but first they migrate to regional lymph nodes and through the thoracic duct to the systemic circulation. If they are IgA-producing cells, they tend to migrate or home to the lamina propria of the intestine and breast, where they are recognizable as plasma cells. Small numbers of cells producing IgM and IgG may also be found in the lamina propria of the intestine, but most of these cells are located in other lymphoid aggregates, such as peripheral lymph nodes and the spleen.

Antibody responses in serum and intestinal fluids differ. The first serum antibody to appear is IgM, which can be detected in hours or days following exposure to an antigen, and which persists for weeks. IgG can be detected in days or weeks, and persists for years.

Intestinal antibody is predominantly of the IgA class. Two monomeric units of IgA are joined together by a J chain, and a portion of the dimer that is formed is wrapped by the secretory component as shown in Figure 1 (12).

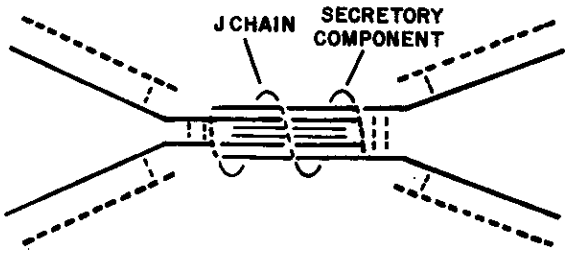

Fig. 1: Secretory immunoglobulin A. Two monomeric IgA units are attached by the J chain. A secretory component is added in the mucosal epithelial cells prior to secretion into external fluids, and increases the resistance of secretory IgA to proteolytic digestion.

This component protects the antibody molecule from proteolytic degredation, thus allowing it to function within the lumen even though antibodies of other classes are digested. Some bacteria produce enzymes that degrade secretory IgA, but *Shigella* have not been shown to do so. Quantitation of antibody in intestinal contents or diarrhoea stools has been difficult, because of variable dilution that occurs in different people and in different disease states. However, in a study of coproantibody in 21 stools from 14 patients with shigellosis, there was a significantly greater concentration of IgA than in 12 patients with acute diarrhoea, but from whom *Shigella* could not be isolated. There was no significant difference in IgG and IgM concentrations, as shown in Figure 2 (13). Additionally, the *Shigella* patients tended to have high titers of anti-*Shigella* coproantibody of all 3 classes, although it was most prominent in the IgA class.

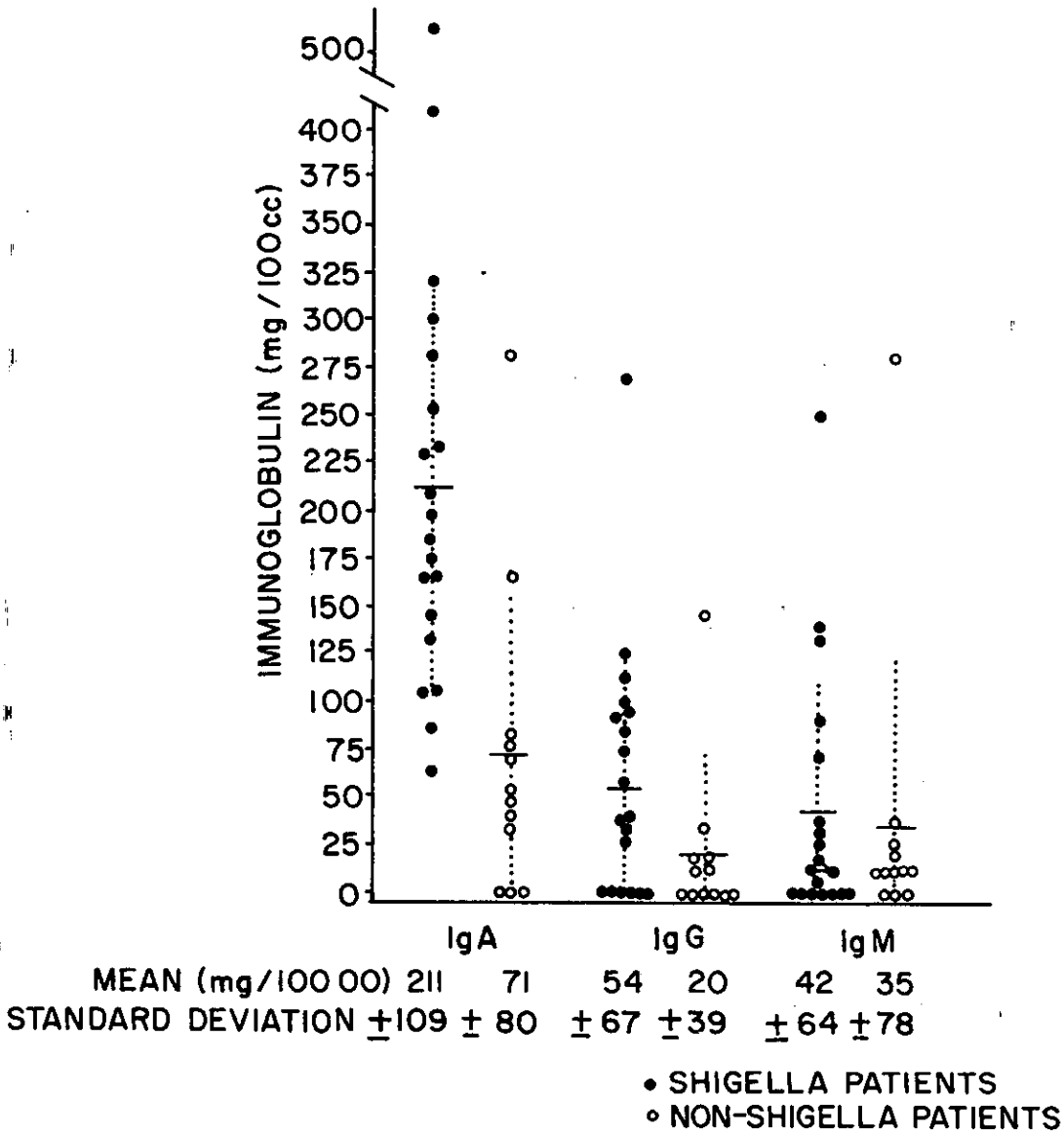

Fig. 2: Immunoglobulin content of *Shigella* stool fluids compared with non-*Shigella* fluids. Results are expressed as mg per 100 ml of fluid phase. The means are represented by horizontal bars, and 1 SD by vertical dotted lines. Note that the IgA concentration is significantly higher (at P=0.01 level) in the *Shigella* group than in the control non-*Shigella* group. The difference is not significant (at P=0.1 level) for IgG or IgM.

PROTECTIVE ROLE OF SECRETORY IgA AGAINST SHIGELLA

Since anti-*Shigella* antibody, especially secretory IgA, appears in the intestinal lumen during shigellosis, studies using the Sereney guinea pig eye test were conducted, to see whether topical application of antibody could inhibit *Shigella* infection. In this study, various immunoglobulin preparations were tested for their ability to protect guinea pig eyes from infection by a virulent strain of *Shigella flexneri*. Secretory IgA was effective in delaying or preventing keratoconjunctivitis in eight guinea pigs when it was used to precoat the organism, and was also placed in the eye with the inoculum (Table 2). Neither IgG nor IgM gave any protection when used in the same way. Protection by secretory IgA appeared to be related to the anti-*Shigella* antibody content of the immunoglobulin, since a low-titered preparation gave less protection than a higher-titered one.

TABLE 2

EFFECT OF PREINCUBATION WITH IMMUNOGLOBULIN ON THE ABILITY OF *SHIGELLA* TO CAUSE KERATOCONJUNCTIVITIS IN GUINEA PIGS

Immunoglobulin used for preincubation	Anti-*Shigella* titer	No. of organisms inoculated (x1.5)	No. of eyes tested	Day of infection in comparison with control			
				same day	1 day later	2 days later	No infection
SIgA	1:8	10^8	4		1	1	2
SIgA	1:2	10^8	2		2		
SIgA	1:8	10^9	2		2		
IgG	1:8	10^8	4	4[a]			
IgM	1:8	10^8	4	4[a]			
SIgA[b]	1:8	10^8	2	2			
No preincubation[c]	1:8	10^8	4	3	1		
SIgA-anti-IgA	1:8	10^8	2	1			

[a] Infection more severe than control.

[b] Organisms washed after preincubation.

[c] SIgA added directly to the eye.

COMPLEMENT

Fresh serum is capable of killing certain strains of *Shigella* (15). Features of this bactericidal system were studied by Wardlaw and Pillemer in 1956, who attributed the killing ability to the properdin system, which was initially thought to function without antibody. However, subsequent investigators, including Nelson in 1958, and Osawa and Muschel in 1960, doubted the significance of the properdin system, and felt that the serum bactericidal reaction is initiated only by antibody and completed by complement.

The term complement is misleading, since it is not a single substance, but rather a group of complex protein - containing molecules that act on one another in a sequential fashion, sometimes referred to as a cascade. An analogy may be drawn between the complement and the clotting sequences, although of course the molecules and their functions are different in the two systems.

Interest in an alternate mechanism to the classical antibody-complement system was revived in 1968, by the observations of Gewurz, Shin and Mergenhagen that endotoxin polysaccharides from Gram-negative bacteria can bypass the early reacting components of complement and still react with the six terminal components. This reaction has been studied in greater detail by Gotze and Muller-Eberhard.

Figure 3 shows the complement components and interactions that are pertinent to this discussion (12).

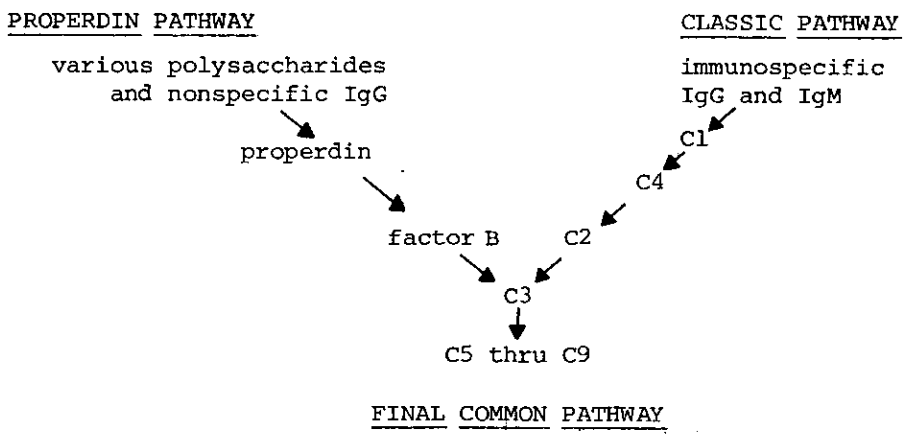

Fig. 3: The complement system. The system is composed of a series of complex molecules that activate one another in a sequential fashion. Either the properdin pathway or the classic pathway may be activated by infecting agents and antibody, and in turn activate the final common pathway. This pathway serves to attract phagocytes (chemotaxis), to promote phagocytosis (opsonization), and to destroy organisms directly (lysis).

The sequence can be initiated by two independent pathways, either of which activates the final common pathway. Regardless of how the final common pathway is activated, it performs three functions related to defense against micro-organisms: the attraction of phagocytes (chemotaxis), the disruption of bacterial cell walls (lysis), and the formation of molecular bridges between micro-organisms and phagocytes (opsonization). Different micro-organisms vary in susceptibility to complement-mediated lysis, but nearly all are susceptible to phagocytes. Those resistant to lysis include most gram-positive cocci and some gram-negative rods; and, not surprisingly, these are the strains that cause most serious infections.

The two initial pathways differ remarkably in their susceptibility to various initiating factors. The properdin pathway, which is also called the alternate pathway, may be activated even in the absence of specific antibody by a wide variety of organisms whose surfaces contain complex polysaccharides. By contrast, the classic pathway can be activated by organisms only after IgM or IgG has reacted specifically with antigen on the organism s surface. Therefore, many invading organisms encounter the properdin-complement system, and are destroyed by lysis or phagocytosis even before specific antibody can be manufactured. Other organisms that do not activate this less selective pathway may not be attacked by complement and phagocytes until after antibody has appeared.

SYSTEMIC HOST FACTORS THAT PROMOTE KILLING OF *SHIGELLA*

The systemic defense mechanisms against bacteria may function in any tissue and in the blood. It is not clear how well they function, or if they function at all, in the intestinal lumen. Therefore, the serum-mediated defenses may only be effective against organisms once they have penetrated the epithelium.

Some strains of *Shigella* can be shown to be susceptible to killing by fresh human serum (16). It is unknown whether this susceptibility occurs *in vivo*, or is a laboratory artifact caused by growth on artificial media. In one study, eight strains of *Shigella* were tested for susceptibility to killing by seven normal human sera (15). Although there was a wide range of susceptibility between strains of bacteria, there was surprisingly little difference in the killing activity of individual sera, and no relationship between antibody titres and killing capacity.

Bacteriolysis required small amounts of antibody, but as little as 0.02 mg of IgM from normal serum restored full killing capacity to 1 ml of antibody-depleted serum. IgG partially restored, but secretory IgA failed to restore the bacteriolytic ability (Figure 4). Both the early reacting classical complement sequence and the alternate C3 activating pathway appeared to participate in killing, as indicated by the roles of C2 and factor B. Killing occurred, but with reduced efficiency, when either of the two substances was missing. However, serum lacking both C2 and factor B could no longer kill *Shigella*. Killing also required the presence of C3, and, presumably, some of the later components of complement are subsequently involved.

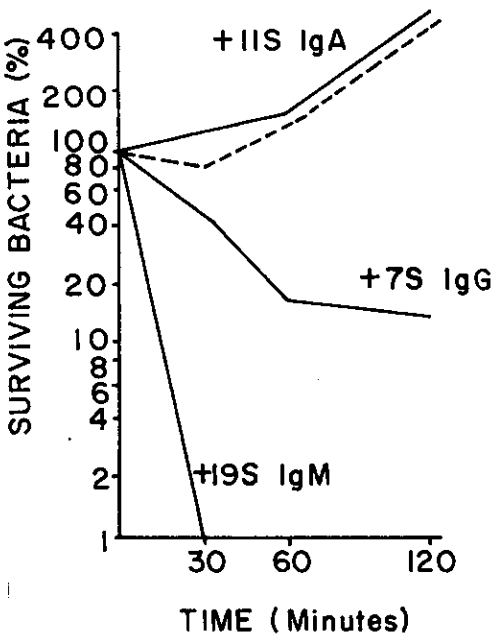

Fig. 4: Effect of immunoglobulin in restoring bacteriolytic capacity of *Shigella*-absorbed serum (dashed line).

It should be noted that even persons with no history of shigellosis have enough antibody that reacts with *Shigella* to be effective in killing serum-susceptible strains. Perhaps this is due to cross-reacting antibody that was stimulated by other related bacteria in the gastrointestinal tract. Once bacteria have penetrated into tissues they encounter not only antibody and complement, but also phagocytic cells. In a study of factors capable of opsonizing *Shigella* (17), twenty-five strains were tested for their susceptibility to phagocytosis and killing by polymorphonuclear neutrophils (PMN). The studies identified several serum factors that could participate in opsonization. The strains varied remarkably in their susceptibility to killing when exposed to heat-labile opsonins and PMN. The heat-stable opsonin was shown to be IgG, whereas IgM was ineffective in the absence of complement, and 11S IgA was never effective. Heat-labile opsonization required immunoglobulin as well as complement, but IgM was the only immunoglobulin demonstrated to participate in this reaction. The quantity of antibody in various sera was important in determining the rate of phagocytosis when each serum was used as a source of opsonin. The alternative C3 activating pathway is required for efficient heat-labile opsonization of *Shigella*, but some opsonization also appeared to occur through the C1-C4-C2 pathway of C3 activation.

MECHANISM OF PROTECTION OF SECRETORY IgA

Since secretory IgA appeared to protect from *Shigella*, at least in the guinea pig eye model, and since the above studies have shown that it does not assist complement or phagocytes in killing *Shigella*, there must be an alternate mechanism through which this type of antibody operates: *Shigella flexneri* 2a were, therefore, tested for their ability to attach to epithelial cells, which had been freed from the large intestine of germ-free mice. Immunoglobulin-containing preparations were tested for their effect on the rate of such attachment. The organisms were tumbled with epithelial cells at a ratio of 30:1.

After 135 minutes, 44% of the epithelial cells had three or more uncoated *Shigella* associated with them, and 6% had more than 10 associated organisms (Figure 5). Organisms precoated with SIgA attached significantly less well

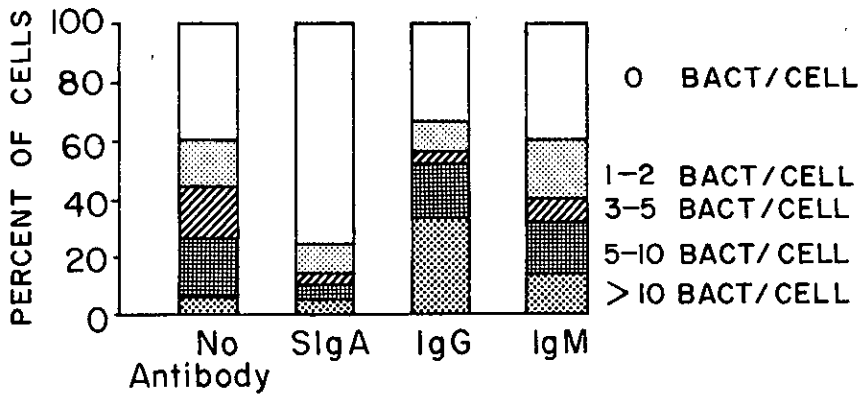

Fig. 5: Percent of epithelial cells from intestine of germ-free mouse that have *Shigella* attached. *Shigella* were either untreated or pretreated with various immunoglobulin preparations. Fewer cells have *Shigella* attached when the *Shigella* have been pretreated with secretory IgA.

with 14% of the epithelial cells having three or more and 6% having more than 10 associated *Shigella*. In contrast, when the organisms were precoated with IgG or IgM, there was no reduction in the number of epithelial cells with organisms attached. With IgG there appeared to be more organisms attached to the cells that did have organisms.

Therefore, SIgA appears to inhibit attachment of *Shigella* to intestinal epithelial cells, while IgG tends to promote such attachment. Inhibition of attachment may be the major mechanism through which secretory IgA protects from shigellosis. Similarly, IgA may prevent some of the manifestations of shigellosis, by reacting with toxin, and preventing their absorption through the intestinal mucosa.

NON-SPECIFIC RESISTANCE TO SHIGELLOSIS

Many studies have shown that colicines produced by normal enteric bacteria may inhibit the growth of *Shigella* (18). However, it remains unproven that this bacterial interference plays any role in natural resistance to shigellosis or in recovery from the disease. Outstanding as evidence that it may play a role are the studies by Formal *et al* (19), that show germ-free guinea pigs to be highly susceptible to septicemia and death following oral feeding of *Shigella*. However, after colonization of the intestinal tract with a single strain of *E.coli*, the animals are unaffected by feeding of *Shigella*. This has been taken as a demonstration of the important role bacterial interference plays in the natural resistance of animals to shigellosis.

An alternate explanation for this phenomenon is available through the studies of Crabbe (20,21). He has shown that germ-free animals have very few antibody-producing cells in the intestinal mucosa, but following the feeding of a single antigen the mucosa becomes populated with antibody-producing cells. It may be that only after population with antibody-producing cells is the intestinal mucosa able to prevent penetration by *Shigella*.

This hypothesis has been tested by feeding *Shigella* to three groups of mice and culturing the mesenteric node on day 5 to determine whether significant deep invasion had occurred. The three groups were: 1) germ-free, 2) monocontaminated with *E.coli*, and 3) germ-free mice which had been fed for one month with dead *E.coli* in the drinking water. Consistent with previous studies by Formal, the node was positive for *Shigella* in 17 of 22 germ-free mice fed *Shigella*, but in only one of 11 *E.coli* monocontaminated mice fed *Shigella* ($P<0.001$). As shown in table 3, the node was positive in only 7 of 21 germ-free mice fed dead *E.coli* for one month prior to *Shigella* challenge. There were significantly fewer positive nodes in this group than in the germ-free mice not fed dead *E.coli* ($P<0.01$).

Group of mice to which *Shigella* were fed	Mesenteric Node		Significance by χ^2 test
	Positive for *Shigella*	Negative for *Shigella*	
E.coli monocontaminated	1	10	$p<0.001$
Germ Free	17	5	
Germ Free Fed Dead *E.coli*	7	14	$p<0.01$

Therefore, antigens from dead related bacteria placed in the gastrointestinal tract appear to induce non-specific host resistance to *Shigella* The mechanism of this resistance is unknown at the present time.

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Chapter 21

Shigella Infections and Vaccines: Experiences from Volunteer and Controlled Field Studies

Myron M. Levine

Center for Vaccine Development, University of Maryland School of Medicine, Maryland, U.S.A.

ABSTRACT

Volunteer studies of induced shigellosis have provided critical information regarding the pathogenesis, clinical features, and aspects of the host immune response to *Shigella* infection, as well as providing a means to assess the safety and efficacy of possible *Shigella* vaccines. Such studies have demonstrated that the inoculum required to induce shigellosis in healthy adults is quite low (10^1-10^2 organisms). This helps to explain the epidemiologic observation of person-to-person contact spread of *Shigella*. The sine qua non of pathogenicity of *Shigella* in man was found to be invasiveness for epithelial cells. A single clinical *S.flexneri* 2a infection 10-52 weeks earlier was found to confer significant clinical (but not bacteriologic) protection upon rechallenge with the homologous strain. Immunization with oral attenuated *S. flexneri* 2a streptomycin-dependent (SMD) and mutant hybrid (MH) vaccines conferred protection against experimental challenge whereas another vaccine consisting of an *Escherichia coli* strain bearing *S.flexneri* 2a surface antigens did not.

In field studies in custodial institutions in the U.S.A. with endemic *S. flexneri* 2a or *S.sonnei* disease, SMD and MH oral vaccines were shown to be sufficiently safe, practical and immunogenic. In one institution where infective inocula were believed to be extremely high, SMD vaccines were not protective. Child-to-child transmission of the SMD vaccines and recovery of streptomycin-independent (although non-invasive) revertants following ingestion

of one lot of *S.sonnei* vaccine, events not previously described, were documented in another institution. Use of SMD *S.sonnei* vaccine eradicated *S. sonnei* infections from an institution where it had been highly endemic for a decade.

INTRODUCTION

Shigella infections persist as a public health problem in both less developed and industrialized environments. In developing areas, shigellosis is an important cause of endemic diarrhoeal disease in toddlers and pre-school children, and occasionally epidemics occur involving all age groups, particularly due to *Shigella dysenteriae* 1. In developed countries, shigellosis is endemic in many institutions for the mentally-retarded and psychotic. It is also a problem for short-term travellers to the less-developed world.

Volunteer studies of induced *Shigella* infection have provided important information regarding inoculum size, clinical patterns, pathogenesis, immune response and evaluation of potential vaccines. Similarly, evaluation of *Shigella* vaccines in prospective field trials under natural conditions has led to a recognition of the efficacy and utility of recent *Shigella* vaccines as well as their drawbacks. It is the purpose of this paper to review this information.

VOLUNTEER STUDIES

Early Dose/Response Studies.

The first induced *Shigella* infections described in the literature were carried out by Shaughnessy *et al* (1946), in an attempt to evaluate the efficacy of heat-killed and irradiated parenteral *Shigella* vaccines. These oft-forgotten studies carry several important messages relevant to later work. Shaughnessy *et al* selected a *S.flexneri* 2a challenge strain (FWI) from a half-dozen candidates, based on its low LD_{50} in mice and its extreme sensitivity to sulfadiazine. These workers carried out dose/response studies in volunteers with strain FWI, beginning with a dose of 10^8 organisms. The first studies involved a notable mode of oral inoculation: Three-and-one-half hours after a meal the volunteers were given paregoric; this was followed 30 minutes later by 2.0 gm $NaHCO_3$ in 30 ml of water. Five minutes later the *Shigella* inoculum was given, suspended in one-half pint of milk. Of three groups of four volunteers each given 10^8, 10^9 or 10^{10} FWI organisms in this manner, mild diarrhoea occurred in three; none had fever, dysentery or toxemia. Stool cultures were positive in nine of 12. Because of the lack of definitive clinical illness with FWI strain administered by this method, these workers excluded milk and thereafter gave *Shigella* inocula with two glasses of water. Four volunteers were then given 1.5×10^{10} FWI organisms in the modified manner, but no illness occurred during the subsequent 24 hours. These same volunteers were given repeat inocula of 1.5×10^{10} on two consecutive days and 5×10^{10} on the next day, again without clinical diarrhoeal illness.

Shaughnessy *et al* thereupon fed a mixture of four other *S.flexneri* 2a strains (2.5×10^9 of each strain), as well as gelatin capsules containing *Shigella*-positive faecal material from one of the earlier volunteers. Under these circumstances, clear-cut dysentery occurred. In subsequent vaccine efficacy studies 10^{10} organisms representing a mixture of four *S.flexneri* 2a strains were fed as the challenge inoculum. Shaughnessy *et al* found that parenteral killed whole cell *Shigella* vaccines were quite reactogenic and provided no protection against clinical dysentery.

Later Dose/Response Studies.

Approximately one-quarter century after Shaughnessy's pioneering studies, DuPont *et al* (1969, 1972) re-established a volunteer model of *S. flexneri* 2a infection as a means of assessing the efficacy of candidate *Shigella* vaccines under controlled experimental conditions. In the intervening years, much had been learned about the pathogenesis of shigellosis in animal models and about the virulence properties of *Shigella*. Specifically, work of Formal and co-workers (1965a) identified epithelial cell invasiveness as the critical virulence property of *Shigellae*. Accordingly, DuPont *et al* employed *Shigella* strains whose invasiveness was well-characterized.

Using known strains of well-characterized invasiveness, DuPont *et al* found that an inoculum of 180 *S.flexneri* 2a organisms caused overt diarrhoeal illness in 9-of-36 fasting volunteers when fed in 30 ml of milk without $NaHCO_3$ of antimotility agents (Table 1). These studies first established the notable pathogenicity of *Shigella* organisms when strains were selected based on epithelial cell invasiveness rather than on LD_{50} in mice.

DuPont and Hornick (unpublished data) later found that 500 *S.sonnei* organisms also caused diarrhoeal illness in 25% of healthy adult volunteers. With as few as 10 *S.dysenteriae* 1 bacilli of an epidemic strain, Levine *et al* were able to induce clinical diarrhoeal illness in one-of-10 volunteers, while 10^2 organisms caused diarrhoea in 50% of recipients. In Table 1, dose/response data are summarized for *S.flexneri* 2a, *S.dysenteriae* 1 and *S.sonnei*. These dose/response data from volunteer studies, demonstrating that minute inocula are capable of causing diarrhoea, offer an explanation for the epidemiological observation that shigellosis is typically spread from person-to-person by direct contact.

Clinical Patterns.

In the course of induced *Shigella* infections in volunteers, it was found that the same inocula could cause a spectrum of responses, including asymptomatic infection, watery diarrhoea without fever, and severe dysentery accompanied by high fever, toxemia, tenesmus, abdominal cramps and scanty discharges of blood and mucus (DuPont *et al* 1969, 1972; Levine *et al* 1973). With *S.flexneri* 2a and *S.sonnei*, the incubation period was usually 1-3 days, while with *S.dysenteriae* 1 the incubation was several days longer.

TABLE 1

CLINICAL DOSE RESPONSES OF VOLUNTEERS TO VARIOUS STRAINS OF *SHIGELLA*

Shigella Strain	Inoculum*	Clinical Attack Rate
S. flexneri 2a	10^2	22%
	$5x10^3$	57
	10^4	59
	10^5	58
S. dysenteriae 1 (endemic)	10^2	25
	10^4	33
S. dysenteriae 1 (pandemic)	10^1	10
	10^2	50
	$2x10^3$	70
	10^4	83
S. sonnei	10^2	25

* Administered to fasting volunteers in 30 ml of milk.

It was recognized in experimental infection in volunteers that illness often commenced with malaise and fever prior to onset of diarrhoea, and that dysentery (blood and mucus in stools) when it occurred was the last prominent feature of the illness to appear.

Pathogenesis.

Volunteer studies played a role in helping to elucidate the pathogenesis of shigellosis. The studies of DuPont *et al* (1969, 1972) and Levine *et al* (1973) established that clinical *Shigella* infection would be induced in volunteers, when strains were selected based on their ability to invade epithelial cells. However, when Keusch *et al* (1972) reported that *S. dysenteriae* 1 strains produce an enterotoxin capable of eliciting secretory

effects in isolated rabbit ileal loops, questions arose as to what role this enterotoxin might play in pathogenesis of *Shigella* diarrhoea.

The development of several genetically modified, recombinant *S.dysenteriae* 1 strains by Formal and associates at the Walter Reed Army Institute of Research offered clinical "probes" to help elucidate the role of *Shiga* toxin in pathogenesis of diarrhoea. A recombinant strain, 482-2E-1, was developed, by transferring the xylose-rhamnose segment of *Escherichia coli* into that of a non-invasive one-step colonial mutant strain of *S.dysenteriae* 1. *In vitro* and in rabbit loop studies this strain produced as much *Shiga* toxin as the fully invasive parent from which the colonial mutant was derived. In contrast, strain 482-2E-1 was completely non-invasive in the opiated guinea pig assay and the guinea pig keratoconjunctivitis test (Gemski *et al* 1972; Levine *et al* 1973). Strain 482-2E-1 was fed to 144 volunteers with 2.0 gm $NaHCO_3$ in doses of 10^6 to $5x10^{10}$ organisms. The strain was well-tolerated without untoward effects by all but one individual, and proliferated in the intestine of most volunteers for 7-28 days. In one individual, however, typical *Shiga* dysentery occurred seven days after ingestion of the strain, manifested by fever, abdominal cramps, diarrhoea and stool discharges containing blood and mucus. It was found that in this one individual the mutant-hybrid strain had dissociated and reverted, regaining the invasive phenotype. Organisms recovered from this individual were typical *S.dysenteriae* 1 biochemically, and were positive in the guinea pig keratoconjunctivitis test (Levine *et al* 1974b).

Review of these observations suggested that invasiveness was indeed the sine qua non of pathogenicity of *Shigellae* and that if *Shiga* toxin played a role in pathogenesis of shigellosis it was only after *Shigella* had penetrated mucosal epithelial cells.

Strain 725 is a potassium chlorate-derived mutant of *S.dysenteriae* 1 that remains fully invasive for epithelial cells but has lost its ability to produce detectable amounts of *Shiga* toxin assayed by cytotoxicity of Hela cells in tissue culture. When fed to 10 volunteers (10^4 organisms), this strain caused diarrhoeal illness and dysentery in five individuals. Scores of clones recovered from coprocultures of ill volunteers were found to be negative when tested in HeLa cell assay for *Shiga* toxin. These observations suggested that a putatively non-toxinogenic *S.dysenteriae* strain could cause typical *Shigella* diarrhoea and dysentery. However, a more sensitive assay for *in-vivo* production of *Shiga* toxin is the serological assay developed by Keusch *et al* (1976). wherein sera are tested for the appearance of antibodies capable of neutralizing *Shiga* toxin effects on HeLa cells in monolayers. When pre- and post-challenge sera were tested from volunteers who developed diarrhoea due to strain 725, it was found that significant rises in neutralizing antibody had appeared. This observation suggested that strain 725 was not devoid of *Shiga* toxin, but rather was hypotoxinogenic, producing too little to detect by direct assay *in vitro* but elaborating enough *in vivo* to stimulate an immune response (Keusch *et al* 1976).

A correct interpretation of the microbiologic and serologic results of volunteer studies with strain 725 is once again difficult in the light of more

recent analyses of *Shiga* "toxin" by isolectric focusing and chromatography, which demonstrate two distinct protein peaks associated with HeLa cell cytotoxicity (McIver *et al* 1975; Keusch *et al* 1975). Only one of these peaks, however, is also associated with enterotoxicity (secretory effect in rabbit loop). It is thus presently unclear whether the neutralizing antibody described by Keusch *et al* (1976) refers to antibody directed against the cytotoxin/enterotoxin protein or the purely cytotoxic protein. Until this is clarified, a definitive interpretation of the volunteer challenge study involving strain 725 cannot be put forth.

Infection-Derived Immunity to *Shigella*.

Epidemiologic evidence suggests that repeated exposure confers serotype-specific immunity to *Shigella* (Levine *et al* 1974a). DuPont *et al* attempted to demonstrate this directly by homologous re-challenge studies in volunteers (DuPont *et al* 1972). Fifteen volunteers who recovered from illness following challenge with 10^4 *S.flexneri* 2a 10-52 weeks earlier were re-challenged with the same inoculum and strain. Diarrhoeal illness occurred in 3-of-15 (20%), versus 22-of-39 (56%) individuals who ingested *S.flexneri* 2a without prior exposure ($p<0.025$). Although significant clinical protection was conferred by a single prior exposure to *S.flexneri* 2a, excretion of the organism occurred with equal frequency in the re-challenge (67%) and control (69%) groups. These observations correlate well with results of longitudinal studies in an *S.flexneri* 2a-endemic custodial institution (Levine *et al* 1974a). There it was shown that an intermediate stage of immunity to *Shigella* exists, wherein, following an initial clinical infection, subsequent *S. flexneri* 2a infections were asymptomatic with positive cultures. Repetitive antigenic stimulation eventually led to complete bacteriologic as well as clinical protection.

EVALUATION OF VACCINES IN VOLUNTEERS AND IN FIELD TRIALS

The pioneer work of Formal *et al* (1965a) established the virulence prerequisites common to all *Shigellae*. These include: 1) the ability to multiply in the intestinal lumen, 2) the capability to invade epithelial cells (LaBrec *et al* 1964), and 3) the capability to proliferate within epithelial cells after invasion (Falkow *et al* 1963). Elucidation of these virulence properties allowed a search for attenuated strains lacking one or more of these virulence attributes.

Mutant Attenuated Strains.

Formal and co-workers described smooth colonial variants of a known virulent *S.flexneri* 2a strain, which were readily identifiable by increased opacity (O) under oblique transmitted light in comparison with the smooth

translucency (T) of the virulent parent strain. In contrast to the parent organism, the spontaneously-derived mutant was nonpathogenic in the guinea pig model or guinea pig eye test (LaBrec *et al* 1964; Formal *et al* 1965a). The mutant was found to occur once in 10^4-10^5 cell divisions of the parent strain, bred true on subculture, and did not revert to the T form.

A spontaneously-derived colonial mutant was found to be safe when fed to monkeys in high dosage (LaBrec *et al* 1964), and was protective on subsequent, experimental challenge (Formal *et al* 1965b). When this strain was fed to adult volunteers (DuPont *et al* 1972a), it was similarly well-tolerated in doses of 10^8-10^9. When 10^{10} mutant organisms were fed to 47 volunteers, 45% developed diarrhoea, 34% dysentery, and 32% fever. Revertant organisms which had regained the invasive capacity were abundant in stool cultures.

Istrati *et al* (1961, 1964, 1967a) developed avirulent *Shigella* mutants by serial passage on 2% nutrient agar slants. *S.flexneri* 2a mutant strain T_{32}, denoting 32 passages, has been shown to be noninvasive and negative in the guinea pig eye test(Istrati *et al* 1963; Istrati and Istrati, 1964). Multiple doses of this strain, containing approximately 50 billion organisms, have proved to be safe in adults and large numbers of children (Istrati *et al* 1965, 1967a,b; Meitert *et al* 1973). A controlled field trial of this vaccine in an institution with endemic dysentery demonstrated some evidence of efficacy (Meitert *et al* 1973).

Streptomycin-Dependent Oral *Shigella* Vaccines.

The attenuated *Shigella* strains that have been most exhaustively studied are the streptomycin-dependent (SmD) strains of Mel *et al* (1965a,b, 1968, 1971). SmD *Shigella* vaccine organisms cannot proliferate in the absence of streptomycin; therefore, they are nonproliferating, as well as noninvasive. SmD vaccine strains have been prepared by Mel from several serotypes, including *S.flexneri* 1, 2a, 3 and 4 and *S.sonnei*. The safety of these strains has been demonstrated in healthy adults and children (Mel *et al* 1965a, b, 1968, 1971, 1974a; Levine *et al* 1972; DuPont *et al* 1972a), and debilitated institutionalized children (Levine *et al* 1972, 1974a, 1975). Multiple doses of this nonproliferating strain, containing 20-50 billion organisms per dose, appear to be necessary for immunization (Mel *et al* 1974a; DuPont *et al* 1972b). Approximately 3-6% of vaccines exhibit vomiting within hours of ingestion of vaccine (Mel *et al* 1965a; Levine *et al* 1972). This adverse effect is encountered almost exclusively following the first dose of vaccine and is uncommon thereafter.

Oral *Shigella* vaccines must be administered with some accompanying agent to enhance gastric transit and survival of vaccine organisms. Pretreatment with 2 g of $NaHCO_3$ in water was the standard method for enhancement of survival of vaccines during gastric transit in immunization of adults and some children (Mel *et al* 1965, 1971; DuPont *et al* 1972a). During pediatric studies in the United States, children refused to ingest the unpalatable $NaHCO_3$ solution (Levine *et al* 1972). A vaccine "cocktail" was devised,

containing 4-8 oz. of milk, a small but barely palatable amount of $NaHCO_3$ (0.8 g), and vaccine. This modification was shown to be as successful as the standard 2 g $NaHCO_3$ pretreatment in enhancement of survival of vaccine organisms (Levine *et al* 1972); and all subsequent vaccine studies in the United States employed this method, Mel *et al* (1974a) also adopted the "vaccine cocktail" for pediatric field trials in Yugoslavia.

In early field trials utilizing freshly harvested vaccine in adults, SmD vaccines were shown to be highly protective against natural challenge during one diarrhoeal season (Mel *et al* 1965b, 1968). In later studies involving children, lyophilized vaccine also showed protection, but vaccine efficacy was less than in the earlier studies (Mel *et al* 1971, 1974a). Studies in children in Yugoslavia revealed that primary immunization conferred protection for more than 6 months but less than 12 months; yearly booster doses restimulated protective immunity to a degree comparable to that of primary vaccination (Mel *et al* 1974a).

Studies in the United States with Mel's SmD vaccines in adult volunteers (non-endemic area), revealed significant protective effects from vaccination in challenge studies, but vaccine efficacy was less than encountered among adults under conditions of natural challenge (DuPont *et al* 1972b). Immunized volunteers challenged with 180 *Shigella* organisms exhibited vaccine efficacy of 60%, while among those challenged with 10^4 virulent organisms vaccine efficacy was 50%.

Shigellosis is endemic in many custodial institutions within the United States. Since neither antibiotics nor isolation techniques have been able to eradicate the infections, control by immunological means was suggested, employing oral attenuated *Shigella* vaccines (Levine *et al* 1972, 1974a). Following preliminary studies demonstrating the safety of two types of attenuated *Shigella* vaccine (Levine *et al* 1972), the SmD vaccines of Mel were selected for U.S. Public Health Service field trials because of the considerable experience with them (vaccination of more than 20,000 Yugoslav adults) and their proven efficacy.

In the United States, more than two-thirds of institutional shigellosis is due to two serotypes, *S.flexneri* 2a and *S.sonnei*. Two institutions were selected for field trials, one with endemic *S.flexneri* 2a disease (Levine *et al* 1974a) and the other with *S.sonnei* infections (Levine *et al* 1975a, 1976b).

SmD *S.sonnei* or *S.flexneri* 2a vaccine was randomly given to participants in the vaccine trials. Each group served as a control group for the other, since immunity to *Shigellae* is serotype specific. In the institution with endemic *S.flexneri* 2a infections, attack rates were high and similar in both vaccinated groups, demonstrating no vaccine efficacy (Levine *et al* 1974a). All cases of symptomatic shigellosis occurred within 9 months of vaccination; and despite clinical protection thereafter, episodic asymptomatic excretion of *Shigellae* was observed in one-third of the children. The level of immunity induced by SmD *S.flexneri* 2a oral vaccine was insufficient to prevent

disease in this institutional environment. Presumably this was due to the primitive level of hygiene existing in a coprophagic custodial population, that resulted in ingestion of "unnaturally large" inocula capable of overcoming the local intestinal immunity induced by the vaccine.

In the *S.sonnei* endemic institution, the controlled field trial design was complicated by some unexpected observations. Evidence of *in-vivo* reversion of the *S.sonnei* vaccine to streptomycin-independent organisms was discovered (Levine *et al* 1975a). The revertants, which were associated with one of two distinct lots of lyophilized *S.sonnei* vaccine, were avirulent (noninvasive) and did not cause clinical adverse reactions. Furthermore, it was observed during vaccination in the course of this field trial that significant child-to-child transmission of vaccine strains was occurring (Levine *et al* 1975a). Following immunization in this institution, *S.sonnei* disease essentially disappeared, despite the existence of several chronic asymptomatic carriers of virulent *S.sonnei* (Levine *et al* 1976b). Although it cannot be proven, it was hypothesized that because of child-to-child transmission of vaccine organisms, the controlled field trial more closely resembled a mass vaccination intervention trial. Thus, disappearance of *S.sonnei* disease from the institution could be attributed to the effect of the vaccine.

SmD *Shigella* vaccines are safe and efficacious against low inocula encountered under many conditions of natural challenge. They require multiple doses for primary immunization, and booster doses must be given 6-12 months thereafter. In the face of extraordinarily high infective inocula, as encountered in some custodial populations, they are probably not effective. Immunity is serotype specific. Nontheless, if most *Shigella* morbidity can be shown to be due to just a few serotypes in a captive population (such as military units) exposed to low inocula, SmD *Shigella* vaccines represent a practical control measure.

Mutant-Hybrid *Shigella* Vaccines.

Another type of attenuated *Shigella* organism that has been investigated as potential oral vaccine is the mutant-hybrid (MH) prototype developed by Formal *et al* (1971b). Noninvasive *Shigella* mutants were conjugated with *E. coli* K12 Hfr^+ males. *Shigella-E.coli* hybrids were selected, in which certain parts of the *E.coli* genome were incorporated into the shigella chromosome in stable form. The xylose-rhamnose (Falkow *et al* 1963; Formal *et al* 1965a) and purine E (Formal *et al* 1971a) portions of the *E.coli* genome were selected since they influence the phenotypic virulence of *Shigella* after incorporation. The association is stable; approximately one in 10^9 hybrids spontaneously dissociates. Even if this should occur *in-vivo*, the dissociated mutant is noninvasive and would have to reach high numbers to achieve the probability of reversion to complete virulence. The theoretical impetus for development of MH strains was the desire for a proliferating strain that would require only one or two doses for successful immunization, overcoming a major drawback of the SmD vaccines.

The MH *S.flexneri* 2a strain has the xylose-rhamnose segment of *E.coli* genome incorporated into an avirulent recipient *Shigella* mutant. This vaccine strain proved to be comparable in safety and efficacy to the SmD *S.flexneri* 2a vaccine, when examined in volunteers (DuPont *et al* 1972a,b); it was also shown to be as safe as SmD *Shigella* vaccines when given to institutionalized children (Levine *et al* 1972). Bacteriological studies to investigate vaccine excretion showed that the *S.flexneri* 2a mutant-hybrid vaccine gave no evidence of being a proliferating strain; the percentage of vaccines that excreted vaccine and duration of excretion were similar to those of individuals receiving nonproliferating SmD vaccines (DuPont *et al* 1972a; Levine *et al* 1972). For this reason, field trials were not undertaken (Levine *et al* 1975).

Four mutant-hybrid strains of *S.dysenteriae* 1 were investigated in volunteers as possible oral vaccine candidates during the Central American *Shiga* dysentery pandemic (Levine *et al* 1974b). One strain was unsuitable because of genetic instability. Two other strains were genetically stable but reactogenic, causing diarrhoea or fever. One of these strains was notable in that the *Shigella* recipient had incorporated in its genome both the xylose-rhamnose and the purine E segments of *E.coli* chromosome, but was nevertheless reactogenic. The remaining MH vaccine strain, 482-2E-1, was unique among all MH strains of *Shigella* examined, in that it was a proliferating strain. Following administration, most volunteer vaccinees excreted the strain for at least 7 days after a single oral dose of 10^8 or more organisms; several vaccinees shed the vaccine strain for more than 28 days after a single dose (Levine *et al* 1973, 1974b). A total of 144 men was fed strain 482-2E-1. Of these, 143 had no adverse reaction. In one volunteer given a $5x10^{10}$ organism dose, the MH strain completely reverted, and the man developed *Shiga* dysentery (Levine *et al* 1973). Organisms recovered from this individual could not ferment xylose or rhamnose and were invasive. Despite the genetic breakdown of the 482-2E-1 strain, it was an important landmark in oral *Shigella* vaccine development, because it demonstrated that a proliferating attenuated *Shigella* strain could indeed be prepared.

E.coli Bearing *Shigella* Surface Antigens.

Formal *et al* (1970) prepared another generation of oral *Shigella* vaccine candidates by genetic manipulation which had theoretical advantages over all previous oral *Shigella* vaccines (Levine *et al* 1977a). In this instance, *Shigellae* and *E.coli* were conjugated; owever, in contrast to the MH vaccines, the new strains utilized *E.coli* as the recipient (female) in conjugation experiments with an Hfr *S.flexneri* 2a (Formal *et al* 1970). It was found that loci for *S.flexneri* 2a group and type-specific surface antigens were associated with his^+ and pro^+ loci, respectively. *E.coli* hybrids containing his^+ and pro^+ markers agglutinated in group- and type-specific antisera. These hybrids offered the theoretical advantages of complete safety, immunogenicity, and propensity to proliferate.

E.coli hybrids bearing *S.flexneri* 2a surface antigens were fed to volunteers and found to be nonreactogenic. Furthermore, evidence of proliferation was seen in excretion studies. Several groups of vaccinees and comparable

numbers of control volunteers were involved in challenge studies with virulent *S.flexneri* 2a. However, the vaccine (multiple doses given in freshly harvested form) failed to protect against either high (10^4) or low (10^2) inocula of virulent organisms (Levine *et al* 1977).

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Discussions

Dr. Levine

About 90 cases of *Shigella sonnei* had occurred per year for four years. These disappeared coincident with vaccination. It is possible that suddenly, at that particular time, the entire population became immune, due to previous disease over four years. However, this seems unlikely. Considering the number of children and cottages, the incidences at that institution were sporadic. However, there were still 40 to 80 incidences-per-year, for four years. Typically, *sonnei* incidence varied considerably from month-to-month. Then something happened, and *sonnei* disappeared for two years. Carriers still existed, and were identified among unimmunized children. But they never spread the disease. Why did *flexneri* 6 suddenly take off? Could it have been introduced from outside? I don't know.

Dr. Feldman

You suggested that *Shigella* is an immunizing disease, so that in this group of children of all ages anybody who had had *Shigella sonnei* once or twice was unlikely to get it again. If this were so the only people getting *Shigella* in that institution should have been new admissions.

Dr. Levine

Or those who had not experienced *Shigella* previously.

Dr. Feldman

After 1971, did it continue at the same rate?

Dr. Levine

Yes. There was not a great turn-over.

Dr. Feldman

You are suggesting that in an environment such as Teknaf, children get *Shigella* between the ages of 1-4, much as they do in Shanghai by age two; and that, even if exposed by age 5 or 6, they will not become ill.

Dr. Levine

Yes, that's right.

Dr. Feldman

Would you say that exceptions might occur if children over, say, age 6, were to be exposed to a very large dose. I believe that in Teknaf and other areas it was found that a significant percentage of illnesses occurred in children over the pre-school age.

Dr. Levine

Well, we don't know. If an older person, let us say a 14-year-old, has a *flexneri* 6, you don't know whether that person had *flexneri* 6 in his early years. That person may have had *flexneri* 2a or *flexneri* 3a. On the other hand, I think it's possible that even someone who is relatively immune may ingest such a giant dose occasionally, that he or she will become ill.

Dr. Feldman

How do you know that it is serotype-specific immunity? That is, you never had an opportunity to test the 2A vaccine in any way, against 3, 4, 5 or 6 or *sonnei*. Only from the ones that were *sonnei* plus *flexneri* 3 and *flex* 1 and 2A -- those was the only challenges, the only vaccine you gave.

Dr. Levine

What we do know is that certain serotypes are more common than others. What was clear from the studies was that immunity was serotype-specific.

Children and soldiers immunized with *flexneri* 2A, were significantly protected against *flexneri* 2A, but not against *sonnei* or any other serotype. There was no difference in attack rates between the *flexneri* 2A vaccines and controls with respect to *sonnei* or any other serotype. There were not enough other serotypes in his study 3A, 1A *sonnei;* about 4 or 5. I think it enough to make the point. This would make sense. Dr. Formal, do you know any other evidence that would support serotype-specific immunity?

Dr. Formal

Flexneri 2 would not protect against *flexneri* 1. Vaccine would protect against *flexneri* 1, 2, 3, 4 and *sonnei*, but not against *flexneri* 6.

Dr. Levine

I think this is a very important point with respect to vaccine development and protecting populations, and it relates to epidemiologic studies. You have to know what the organisms and the serotypes are in the community. And even though you look at Leonardo Mata's slide, that shows 12 different serotypes, in fact, only 4 or 5 account for more than half the infections. That means that if you have only 4 or 5 antigens, theoretically you could reduce morbidity by half.

Dr. Reed

One of the things I think we ought to look at is how *Shigella* epidemics occur. Perhaps they tend to occur in malnourished people, perhaps in people who tend to have an impaired complement level, or impaired antibody level, I think all these things ought to be studied. I think, too, one might well study the immune response to infection with shigellosis or perhaps vaccine challenge, not only in healthy, well-nourished individuals, but also perhaps in individuals with marginal nutrition, people who perhaps tend to get more severe diseases.

Lastly, I think there is no up-to-date information about the host-defense to the inter-cellular location of the organism within the intestinal epithelial cells; and I would think it is clearly in order to try to determine whether or not the host has any defense, and, if so, what this defense consists of. I think that probably is a hard thing to approach and, off-hand, I do not know what kind of a technique one would use. However, with some thought one might come up with some new approaches. It would also be important to look at the causes of bacteremia in shigellosis, as, theoretically, from considerations in my presentation, it should not occur.

Dr. Formal

Apropos of Dr Reed's comments, we are doing an experiment. Using 60 monkeys, we immunized 30 of them and left the other 30 as controls. We challenged 15 immunized monkeys and 15 control monkeys. And we got very severe disease among the control animals, but virtually no disease among the immunized monkeys. Then, on day 2, we sacrificed the other 30 animals, half of which were immunized and half were controls. This was done at the peak of the disease. We cultured the lumen of the bowel. We found equal numbers of organisms in both the immunized and control animals. Then we did pathology and fluorescent antibody tests on the 30 sacrificed animals. In all the control animals, we found histologic changes in the colon and invasion. In the immunized animals, there was no evidence of invasion; and even though there were large numbers of organisms in the lumen, there was very little pathology. So the only thing we can say is that we got immunity by blocking invasion. And yet even in those few animals, where we got a small foci of infection which we could not tell by fluorescent antibody, it was assumed by histology that we were getting some type of cellular immune response that was combatting the invasion.

Dr. Kabir

The problem of immunity of *Shigella* is very complex. We do not know the duration of immunity. Nor do we know how long the bacteriocidal effect of the serum lasts.

Dr. Feldman

A bacteriocidal effect even in non-immune individuals was evident in this study. So it would be necessary to separate what results from immunity from what the baseline is. The duration of the gut response is a very important question to answer.

Dr. Formal

I think we have one product ready for testing -- approximately 7-to-10 thousand doses, on which we hope we will be doing safety testing within 6-12 months, depending on the paper work and red tape involved. The first two studies will be done on 15 people each and then we will go on from there. I think if we are lucky we will have, very soon, a *flexneri* 2 and *flexneri* 3 vaccine. We have worked on these mainly for selfish reasons, because of the fact that *sonnei*, *flexneri* 2 and *flexneri* 3 account for about 99% of *Shigella* in the U.S.A. I think that if we succeed with two, it will not really be a problem to formulate vaccines for the other serotypes.

We already have succeeded with *E.coli* in transferring *Shigella* antigens of type 1 from six, from *Shigella* to *E.coli*. With *Salmonella*, its a difficult problem, but I don't think it is insurmountable. I think we will be able to do that. The thing which has eluded us in the past, though we have tried, is to get a *shiga* vaccine. We have not been successful in transferring *shiga* material from *shiga* antigens. However, I just talked to Mike Levine, and will be trying different techniques which probably will be successful. That, I assume, is going to be a longer term project.

Dr. Kostrezewski

The conclusion is that you have some vaccines ready for testing, which are particularly oriented against the epidemiological situation in the United States. *S.sonnei* is not of great importance in this country (Bangladesh). Here, *S.flexneri* type 2 is a problem, so we could think that after the preliminary work is done there, something appropriate for Bangladesh could be developed.

Dr. Aziz

The thing that is most needed for Bangladesh is the combination of *flexneri* 2a, 2b along with *shiga*. Also, I would say about *S.sonnei* that, even though it is not a problem now, I visualize that once people become

immune to *shiga* and *S.sonnei, sonnei* may take over. So, to take a precautionary step, all these four things should be in a single vaccine. On what time scale can you produce such a vaccine?

Dr. Formal

I think the vaccine we have against *flexneri* 2a is capable of protecting against *flexneri* 2b. We will have two or three to protect against *sonnei* within a year or more. I just cannot say this for *S.shiga*. It may be we will use a different procedure.

Dr. Rahaman

I am not quite familiar with the literature of *Shigella* immunology in the community. Dr. Shahjahan Kabir is here. He is working with the cholera antigen, particularly the outer membrane antigen. I would like to invite Dr. Kabir to tell us whether a similar methodology should be used in research on shigellosis.

Dr. Kabir

As far as the outer membrane protein of *Shigella* is concerned, I think the best person to comment would be Dr. Samuel Formal, because in the November issue of *Infection and Immunity*, there was a paper, "Outer membrane proteins of *Shigella* spp." from a Polish group. Prof. Romanowska claims to have achieved *Shigella* protection in her studies, by immunization with outer membrane proteins. Dr. Samuel Formal is very much aware of the paper. I think the paper looks very promising. We could pursue research along that line. We have an abundance of strains. It is quite possible to do such studies.

Dr. Formal

Prof. Romanowska extracted protein or possibly a series of proteins from the cell wall of immunized guinea pigs parenterally; and showed that this vaccine protected them against a large challenge -- both against a homologous strain and a heterologous strain. It might have been a *flexneri* and *sonnei* challenge. She showed that the protection was greatest against the homologous strain and somewhat less against the heterologous strain. This is a different type of result from what one gets when one uses an oral vaccine. But this does not preclude the possibility that an antigen isolated from the cell will act differently when used as an immunogen. So I would certainly have something to look forward to. The protection was against a severe test challenge, which is very strange because the eye is not protected by circulating antibodies which ordinarilly do not penetrate it.

Dr. Kostrezewski

We should recommend the continuation of the work on the vaccine against shigellosis. We cannot now put any target date.

Chapter 22

Intervention of Shigellosis by Hand Washing

Moslemuddin Khan

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

ABSTRACT

Secondary infection within families of clinical cases of *Shigella* in Dhaka ranged from 20%-25%. In hospitalized cases the mortality rates for severe cases were 5%-8%, 10-15 times higher than in cholera, indicating that simple rehydration is inadequate. In view of high attack rates, increasing resistance, high mortality and the reality of very poor sanitation in heavily populated areas of Dhaka city we decided to study the impact of a simple intervention (no changes in the environment or in the community) on the disease. Hand washing with soap and water was chosen as the simple intervention.

Culture-positive cases were selected from the hospital for a 10-day follow-up. Controls matched for age, socio-economic status and neighbourhood were selected. The study families were provided with 2-4 pieces of soap and 2-3 earthen pitchers. Members of all the study families were advised to wash hands with soap and water after ablution and before meals. Compliance was checked by observing size of the soap and water usage. Rectal swabs of family members were collected daily for culture.

The overall secondary infection rate in the study group was 10.4% and the control group 32.4%. The secondary case rate in the study group was 2.2% and in control group 14.2%. These results suggest an important effect of a simple and inexpensive intervention, that is easily understood and implemented by families even under unsanitary environments.

INTRODUCTION

In a family study in Dhaka it has been shown that 13.3% of contacts of patients with *S.dysenteriae* type 1 developed symptoms (1). Gangarosa *et al* had shown that the secondary attack rate in Guatemalan villages was 36.5% for males and 30.5% for females (2). Ceaser A, Mendizabal *et al* reported that fatality due to shigellosis was 4.8% in villages and 10%-15% in acute hospitalised cases (3). The mortality rate in the ICDDR,B treatment centre was 5%-8% (unpublished observation). This rate is 8-to-12 times higher than the mortality rate of cholera or other watery diarrhoeas. In an outbreak on St. Martin Island, Bangladesh the mortality rate was 6.4% (4). Thus, death due to shigellosis, even under institutional treatment, is among the highest in diarrhoeal diseases.

There have been several reports stating that shigellosis spreads through water (5,6,7). The setting in Bangladesh favours water-borne illness.

Preventive measures have not been effective against the spread of *Shigella*. Vaccination has not been found promising (8). Antibiotics could be used prophylactically, but resistance in *Shigellae* is increasing. In 1968, broad spectrum antibiotics were quite useful in treating shigellosis in this country (9). This is not true now, when many *Shigellae* are resistant to most antibiotics. Many investigators from different parts of the world have observed that resistance of *Shigellae* to multiple antibiotics is present. If no new effective drug is developed, a day might come when all available drugs would be ineffective in killing *Shigellae*. This will create grave problems for the developing countries, where the standard of health and hygiene is very low.

Simple hand washing has been effective in preventing acquisition of potentially pathogenic bacteria in infants (10). Although the evidence is not rigorous, hand washing with soap and water may prevent nosocomial infection (11).

We decided to use this simple procedure, providing soap and water containers to family contacts of *Shigella* patients to wash hands, to prevent the spread of the disease. Education of the families and surveillance by a health worker was necessary to ensure that people did wash their hands after defaecation and before eating or preparing foods.

METHODS

Stools of patients attending the ICDDR,B outdoor clinic with diarrhoea/or dysentery were cultured using standard techniques (12). Age, sex and socio-economic status (SES)-matched cases of shigellosis were selected alternately as study and control groups. The pre-conditions were that the patient was willing to cooperate and had a family living within the city

or the suburbs. Rectal swabs of all family contacts, including controls, were obtained daily for 10 days for culture on SS and MacConkey media. Sensitivity tests were done on Muller-Hilton plates using standard techniques.

Hand washings were also obtained for culture in gram-negative broth (G.N. broth). Domestic water was cultured after millipore filtration. Histories including SES, water use pattern, sanitation facilities and illnesses were obtained. Contacts yielding the same types of isolates as the index cases were termed as secondary infections. These infected contacts passing three or more diarrheic/dysenteric stools in 24 hours were termed as secondary cases. Significance was measured by chi square tests.

The fifty study families were provided with 2-4 pieces of soap and 2-3 earthern pitchers for storing water, depending on the size of the family. They were urged to wash hands with soap and water after passing urine or stool and before serving, preparing or eating any food. The size of soap and amount of water used were checked daily. Reports on diarrhoeal illness were also obtained from the individuals or the parents in case of children. The fifty control families were not given either soap or pitcher and education was not provided. A further 50 families who were provided with soap only and another 50 with water containers only were also studied, to compare the efficacy of soap and water independently. Vitamins and oral rehydration salt in cases of avitaminosis and diarrhoea were supplied, and severe cases were taken to the ICDDR,B Treatment Centre.

RESULTS

Essential comparability of the groups studied are presented in Table 1. Those who collected drinking water from tap or hand pump tubewells ranged from 92% to 98%. However, those who used mixed sources of water (river, canal, pond) varied from 20% to 32%. The use of sanitary latrines ranged from 36% to 56% between the groups.

The age-specific subsequent infection rates among the family contacts are shown in Figure 1. The overall infection rate was 10.1% for the study group and 32.4% for the control group. Total difference was highly significant.

The overall secondary case rate was 2.2% for study and 14.2% for control group. The overall reduction in secondary case rate was 84%. The difference was highly significant.

The efficacy of the intervention in the groups affected with different types of *Shigellae* is seen in Figure 2. Hand washing significantly reduced subsequent infections and case rates in all types of *shigellae* except *S. dysenteriae* type 1. *S.flexneri* could be reduced by 67%, *S.dysenteriae* type 1 by 33% and other *Shigellae* by 87%. Significant differences persisted, even if the possible co-primary positive isolates were excluded.

TABLE 1

COMPARABILITY OF STUDY AND CONTROL GROUPS

Groups	Household population excluding index	Percent <5 yrs.	Average room/ family	Average member/ family	Average member/ room	Drink Tw/Tap water	Used mixed water	Used sanitary latrine
Study (soap + water)	279	23.3	1.56	5.6	3.1	94%	26%	36%
Control (no soap) (no water)	318	24.8	1.78	6.4	4.0	98%	20%	42%
Only soap group	300	21.6	1.58	6.9	3.7	92%	32%	50%
Only water group	299	23.1	1.72	6.0	3.3	93%	28%	56%

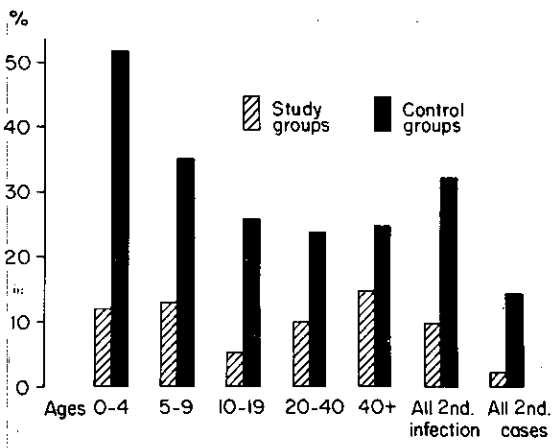

Fig. 1: Age-specific subsequent infection rates in study and control groups in percent

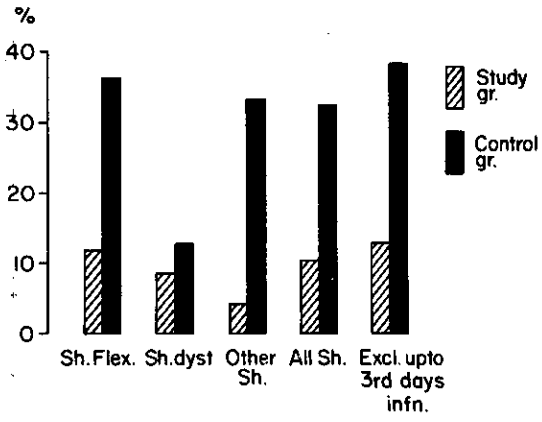

Fig. 2: Secondary infection rates in study and control groups by types and percent of *Shigellae*

The quantity of water used for domestic purposes per-person per-day is related to infection rates (Table 2). The study group used less than 4.5 kg. of water per-day per-person for drinking and cooking and had an infection rate of 8%, as against 4.2% among those who used more than 5.5 kg. per-day per-person (N.S.). These rates were 34.6% and 30.0% in the control groups (N.S.). Those study families who used less than 20 kg. of water per-day per-person for bathing and washing had an attack rate of 14.6%, as against 1.5% among those who used over 25 kg. of water per-person per-day (significant). Those controls who used less than 20 kg. had an attack rate of 22.1%, as against 14.3% in those who used more than 25 kg. of water per-person per-day (N.S.).

Rates of infection in families who were provided with only soap or water pitchers are shown in Figure 3. The *Shigella flexneri* secondary infection and case rates for the soap-using group were significantly lower than the water-using group ($p<.01$). There was no significant difference for *S.dysenteriae* type 1 in this regard. There was, however, a suggestive difference for other *Shigella* species.

None of the 579 study and soap individuals whose left hand washings were cultured was positive for *Shigella*. Out of the 617 control and water group individuals, seven were positive for *Shigella* ($p<.025$). Additionally, among the 432 sources of household water tested, two were culture positive for *Shigella*, one from each group.

In order to estimate the probable prevalence of shigellosis in the community we chose to analyze the non-intervention groups (control and water only). By looking at the contacts' frequency of isolation of *Shigella* species, other than that found in the index case, we can estimate community prevalence. The infection rate was 5.8%, of whom 1.9% developed symptoms of dysentery from these two non-intervention groups (Table 3).

TABLE 2

QUANTITIES OF WATER USED BY THE STUDY AND CONTROL GROUP AND RATES OF SECONDARY INFECTION

Average quantity of water	Study			Control		
	Secondary infection rate			Secondary infection rate		
	Contact No.	Infection No.	%	Contact No.	Infection No.	%
Drink average kg < 4.5	25[a]	2	(8.0)	101[e]	35	(34.6)
and						
Cook average Kg 5.5 +	165[b]	7	(4.2)	50[f]	15	(30.0)
Bath average kg < 20	41[c]	6	(14.6)	95[g]	21	(22.1)
and						
Wash average kg > 25	66[d]	1	(1.5)	21[h]	3	(14.3)

a vs b N.S. e vs f N.S.

c vs d Significant g vs h N.S.

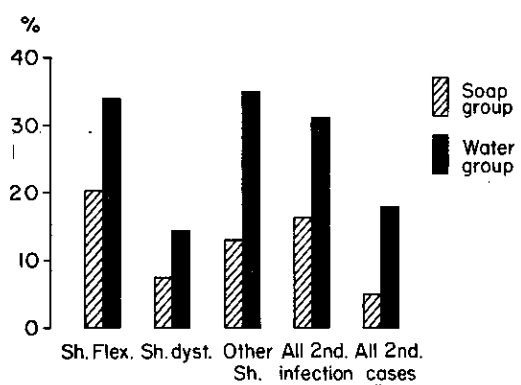

Fig. 3: Secondary infection of shigellosis in groups who used only soap and only water in percent

TABLE 3

SHIGELLAE OTHER THAN INDEX, IN CONTROL AND EXCESS WATER USING FAMILIES

Groups	Contracts No.	Infection No.	%	Case No.	%
Control	268	15	(5.6)	5	(1.9)
Water users	249	15	(6.0)	5	(2.0)
Total	517	30	(5.8)	10	(1.93)

DISCUSSION

Although the rate and problem of shigellosis varies from country-to-country according to the hygienic standard, it has not been eradicated even from developed countries. According to this study, which also confirms our previous study (1), we found that in any given 10 days time, 1.93 persons per 100 are sick with shigellosis different from the *Shigellae* of the index case. According to this finding, annually every 100 persons suffer from 70 episodes of shigellosis in addition to hospitalized ones. We have also found that in any given 10 days time, 11.2% of people suffer from diarrhoeas of unknown origin, which means 40% additional episodes of diarrhoea per 100 persons per year (1). In addition, there are cholera (0.3%), rotavirus, ETEC and campylobacter prevalent in our community. It is not hard to see why over 20% of deaths, especially of children, are claimed by diarrhoeal diseases alone.

Practical and immediate solutions are needed when proper sanitation is not feasible and vaccination does not help. Antibiotics are expensive, and changing resistance makes these even less useful. People are forced to use surface water which transmits the organisms of diarrhoeal disease. Hand pump tubewells for drinking alone did not help in reducing diarrhoeal diseases and cholera, since other sources were used for bathing and washing (13,14).

Hand washing with soap seems to be an effective and practical alternative. In this study we have seen that an additional quantity of water does not help much. Washing hands with soap, however, markedly reduces the secondary infection and case rates. It is most effective in younger groups, possibly because mothers feed them. Adults often go out and do not have soap available for washing. The effects were most significant in *S.flexneri* and other *Shigellae*, except *S.dysenteriae* type 1. Failure in case of *Shigella dysenteriae* type 1 might be due to its greater virulence and smaller dose requirement

for infection (15). Diarrhoeas due to other causes were also reduced (37%) with the use of soap and water.

Out of the 7 hand culture-positive contacts, 3 developed symptoms. Transmission of shigellosis from the left hand acquired by washing buttock to food and the right hand is quite easy. Two samples of household water also yielded *Shigellae* (.60%).

The health education imparted was most simple and easily understood by everybody. They were advised to wash hands with soap and water without fail, once after every ablution following defaecation, and once before taking or serving every meal. The national health education programme can publicize it through popular media.

The approximate cost for treatment of one case hospitalized with shigellosis is shown in Table 4. Though shigellosis is said to be self-limiting, yet it claims 8%-15% of all hospitalized cases, in spite of best institutional treatment (3). Even though only one out of every five symptomatic

TABLE 4

TREATMENT COST FOR ONE HOSPITALIZED CASE OF SHIGELLOSIS

Average days in hospital and expenses	Transport cost/ patient	Visitor's transport cost	Attendant's wage loss	Patients wage loss	Grand total
7 days Ta.1050.00	2 trips x 20 Ta. 40.00	20 x 7 days Ta. 140.00	20 x 7 days Ta. 140.00	Taking 1/5 as earning @ 20 day Ta. 28.00	Ta.1398.00 us$=90.00

cases needs treatment, the annual saving of a family from intervention comes to a significant amount (Table 5). In addition, we expect that, by adopting this practice in families, nearly 80% of the hospitalized shigellosis cases and 37% of other diarrhoea cases would be reduced. But soap should be readily available and cheap. The practice needs to be implemented in countries where shigellosis is a problem.

TABLE 5

ANNUAL SAVING PER FAMILY BY ADOPTING INTERVENTION TECHNIQUE

Group	No. of secondary cases in 10 days	No. of other shigellosis in 10 days	Expected annual cases in 100 families	Expenses for treatment of cases of 100 families	Annual expenses for intervention of shigellosis in 100 families	Net annual saving per family
Study group 100 families	17	4	(17 + 4) X 36 = 756	Ta. 756X1398 = Ta.1056888[a]	Ta.20 X 100 X 36 Ta. 72000[c]	(b-a-c)÷100 Ta.35013 = US $ 2260
Control group 100 families	82	10	(72 + 10) X36 = 3312	Ta. 3312X1398 = Ta.4630176[b] = US $ 298720	-	-

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Discussions

Dr. Keusch

Shigellosis is really an interesting disease. For any intervention that is made, there are studies that demonstrate a decrease in the rate of shigellosis. Almost anything works. The real question is what is the major factor in transmission? All my friends involved in epidemiological studies tell me that shigellosis is a disease spread by person-to-person contact. If that be true, tell me what is the important factor to consider epidemiologically? Should we worry about files or do something else?

Dr. Khan

In my study of intervention I found that those people who were supplied with a cake of soap and a water pitcher to store water for washing hands, had a much lower infection rate. We don't know whether it was the effect of the soap or the water that made the difference. To examine whether water alone was as effective as soap, we took another 100 families. Fifty families got water pitchers only for storing water and 50 got soap only. We found that infections among people who used only water or only soap were not reduced; but for those who used water and soap, the results were very conclusive. Their infection rate was significantly less than for those who used water only. During the first epidemic of *Shigella* dysentery in the St. Martin Island, which Dr. Rahaman has already described, we were puzzled to see that it took 2 months to spread the disease over the 3-mile-long island. There was no common source of water, no festival, in fact nothing in common. The only possible carrier was the fly. And there was an enormous fly population, so large that it was almost impossible to sit without moving constantly, swatting flies. We finally concluded that it was the flies which carried the infection from one end of the island to the other end. Maybe that is the reason it took such a long time.

Chapter 23

A Study of the Interpersonal Spread of Human Faeces in Rural Teknaf of Bangladesh

K.M.A. Aziz, K. Zahid Hasan, Y. Patwary, M. Mujibur Rahaman and K.M.S. Aziz

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

ABSTRACT

Transfer of human faeces among people was observed by following the normal regular movement of hands of the mother, particularly after defaecation or after cleaning of the bottom of a child. Only those aspects of hand movement were observed which were likely to play an important role in transmitting faeces among human beings. The movement of the hands of 21 mothers with children aged under five years was observed. These mothers were from 21 different households in several villages located within the Teknaf *thāna*. The main objective of this study was to gather first-hand information in order to develop health messages for community members to promote use of water following the cleaning of bottoms after defaecation.

The study explored the possibility of contamination of food and water from human faeces through the use of naked hands in cleaning faeces after defaecation.

INTRODUCTION

Human faeces act as the principal source of enteric pathogens. The purpose of this study was to identify the various ways of spreading enteric pathogens through human faeces within the household and the community in rural Teknaf. Identification of these methods may provide useful clues for development of a strategy for health education, in order to reduce transmission of diarrhoeal diseases. Cleaning the anal region after defaecation, with water, sticks, leaves, grass, etc., is common practice in Asia. The unwashed or inadequately washed hand may act as the main vehicle for spreading diarrhoea-causing organisms.

Isely (1) (n.d.:1) observed that when the environment is contaminated by the stools of infants and small children, multiple sources serve as the vehicle of contamination. All the main pathogens causing diarrhoea are transmitted from anus to mouth (2). There are many opportunities for such transmission in a poor and crowded community. To identify the mechanism of transfer of human faeces, it was decided to observe the hands of the mother after defaecation or after cleaning the anal region of a child.

The study villages are situated in Teknaf *thāna*, a coastal area situated in the southern tip of Bangladesh. The people in this area use water from ditch or tubewell for all purposes. Most of the study households had no fixed latrines. The study population is Muslim and the major occupation is agriculture and fishing. Most of them had no formal education.

Shigellosis is known to be associated with poor hygiene. A single intervention, like washing of hands with soap and water, sharply reduces the rate of incidence of this disease (3). This study showed that secondary infection rate of shigella was 10% in the study group and 32.4% in the control group. This study clearly demonstrated the importance of hand washing in reducing the rate of *Shigella* infection. Ash could be used when soap is not available (4).

In the Teknaf study area people carry a pot of water when going for defaecation (5). This water is used for washing the anal region with the left hand after defaecation. Frequently, the amount of water carried is not sufficient for proper cleaning. Due to scarcity of water stored at home, most often hands are not properly washed before eating or handling food. Because of *pardā*, women rush through defaecation, particularly during the day-time. So there is little scope for proper washing of hands afterwards. As women are the carriers of water, their restricted movements during the day, due to *pardā*, result in limiting the quantity of water stocked at home.

METHODS

The data for this study were collected through participant observation method. Two female field workers were assigned to collect information on movement of hands after defaecation, particularly in relation to washing of clothing, utensils and food items before cooking of food, handling of water and interpersonal contacts. Transfer of human faeces among people was observed by following the normal regular movement of hands of the mothers of children under five years of age, after defaecation or after cleaning of the anal region of a child. Length of each of these observation was more than 12 hours, beginning before sunrise. The prospective mothers were asked permission by the female field workers to be allowed to stay within their respective houses to observe the activities centering around their children for a day. Only willing mothers were the subjects of observation. Mothers from 21 different households belonging to several villages located within the Teknaf *thāna* volunteered, However, 4 mothers refused. The two female field workers were instructed to record all activities of the mothers, with special emphasis on behaviour after defaecation by themselves or by their children.

FINDINGS

The findings of this study were that contamination is transmitted through fingers mainly in the following ways:

1. Handling of utensils: through handling cups and other utensils before serving.

2. Serving of food: especially by direct touch. Most of the time only the right hand is used; but in some cases, e.g. making or handling rice cakes or pickles, both hands are used.

3. Serving of fruits: while peeling or serving fruits, such as mango, pineapple, papaya, jackfruit, etc.

4. Feeding infants and children and sharing infant's food: by holding the breast before breastfeeding, feeding the baby with fingers, sharing of food from the same plate by the mother or other adults and children, eating child's left-over food.

5. Carrying and storing water: while drawing water from a well both hands are used, thereby transferring contamination from the hands to water via rope. When water is transferred from the well or from tubewell to the pitcher, the fingers of both hands frequently touch the collected water, When the collected water is poured into other containers, the fingers of both hands also come in touch with the water.

6. Disposal of the faeces of children: the soiled pieces of clothes or mats are cleaned in water with both hands, Infant and children's faeces are frequently disposed with either an oyster shell, or broken tin plate or leaves with both hands. Following the removal of faeces of a child from the courtyard, the spot is cleaned with the help of a broom, which is subsequently used again to clean the floor of the dwelling house without washing it. After the removal of child's faeces from the floor of the dwelling house, the mother frequently uses her foot to clean the spot, pouring water until the faeces is no longer visible. Subsequently at the prayer time, while undergoing ablution *(azu)*, she touches the same foot to wash three times by using her hands.

7. Preparation of food: especially while making 'mashed preparations' *(bhartā)* out of cooked brinjal, boiled potatoes and dry fish; tasting the curry or other food preparations by putting a bit of it on the palm of the left hand.

8. Preparation of betel leaf with betel-nut: betel leaf and betel-nut are touched by fingers of both hands before chewing.

9. Personal habits of washing herself and the child: cleaning of teeth by using the index finger of the right hand and taking of powder for cleaning from the palm of the left hand, washing of hands and face of child by using both hands. The water pot used to hold water for cleaning of anus after defaecation is also used for ablution *(azu)* and routine washing of hands and face for self and others.

In addition, it was also observed that the riverside, which was full of faeces, was repeatedly being washed by the high tide, and during such process of washing the children were found to play in the waves of the rolling water.

However, certain routine activities were found that might be helpful in reducing or stopping the transfer of human faeces among people. These included washing of clothing, taking of bath using soap, cleaning of utensils by using ash and straw, washing of hands by using earth after defaecation.

DISCUSSION

Presence of faeces on the outer part of the body or in items of food or drink is universally viewed as impure *(nāpāk)* in Teknaf. After defaecation, the cleaning of the anal region is done exclusively by using the bare left hand. Left hand is therefore consciously avoided in many activities during a person's daily life. The specific avoidances include salutation or greetings, shaking of hands, handing over or receiving any objects including food items. In spite of prevalence of such consciousness for avoiding the use of left hand, which is used for cleaning faeces, some activities are being done in the observed households which are responsible for transfer

of faeces unintentionally. Incompletely washed faeces-soiled hands used in preparing or serving various eatables, washing hands and face of others and self, and drawing of water from wells facilitate the transfer of faeces.

It was found that frequently people were conscious about the presence of faeces so long as it was visible and, accordingly, when soiled clothes were washed after the removal of the faeces, both the hands were used to clean them to acceptable levels. In the past, many of the investigators who studied the sanitation problems did not examine the role of inadequate handwashing after defaecation in transferring the faeces (6). However, Isely (n.d.:1), quoting the Academy for Educational Development, (7) observed that micro-organisms are transmitted via contaminated fingers of the child or his mother,

- to household objects;
- to food itself;
- to water transported or stored;
- to the hands of other children, and
- ultimately to the mouths of other susceptible individuals.

To maximize the expected benefits of the separation of faeces from the environment by installing sanitary latrines, attention must be given to introduce adequate hand washing after defaecation, so that soiled fingers do not serve as a medium of transferring faeces. The fundamental issue is to understand how people may be motivated to change the age-old custom and wash hands adequately after defaecation. The ways of such motivation can only be developed after proper understanding of the existing ways of transmission of faeces from person-to-environment, from environment-to-person and from person-to-person. This study has given rise to the following research topics regarding the transmission of enteric diseases which deserve further investigation: (a) the role of fingers; (b) the role of kitchen utensils; (c) the role of interpersonal contact of children.

ACKNOWLEDGEMENT

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Discussions

Dr. Keusch

I would congratulate Dr. K.M.A. Aziz and Dr. M.U. Khan for the two papers they presented this morning. These are extremely important studies, especially from the point of view of intervention. We can learn many things about these diseases, but these particular kind of studies have not been done in the past; the data is very exciting.

Dr. Kostrezewski

We listened today to two categories of intervention studies. Targeted were attempts to control shigellosis. One, the group of papers on immunity, especially on vaccination, presents data on quite highly sophisticated work, which, in certain ways, results from about 70-80 years of studies on vaccines. This is the first time we can prove that a vaccine, if properly prepared and properly applied, may be effective. However, there are many problems involved. One important problem is the changing pattern of shigellosis. However, having the possibility of speeding up the work and updating our information on the aetiological pattern and particularly on an effective surveillance system to learn where each type of dysentery is prevalent, will be helpful.

I still think that vaccination offers us some hope at the present time. However, I am afraid that the information we are receiving about the possibility that high doses of infectious agents may be able to overcome

acquired immunity may mean that a vaccine may be less successful in some situations. Particularly disturbing in this regard was the last presentation of Dr. K.M.A. Aziz, about some anthropological patterns, because these may be evidence of very high contamination levels. For this reason, I think the second group of studies is of extreme importance. As a matter of fact, I am speaking about enteric diseases as a whole. They are the diseases transmitted by dirty hands. So, by washing hands the difficulty is overcome. I would say that Bangladesh presents a very good situation for this sort of study, because this a country where there is plenty of water.

At the same time, however, I think we must ask ourselves what will happen if we look at all these anthropological problems in North Africa communities where water is not easily available, and people use a small jar of water for washing for the whole day. This is becoming an important issue, and I think this is the sort of study that should be done in the future. We also should think of doing the type of anthropological studies that may answer some questions about the primitive every day movements of people--movements which contribute to the spread of diarrhoeal diseases, and cause endemic situations and epidemics. I think we should encourage more interest in this direction.

Dr. Sutton

Mothers may play a more significant role in the transmission of *Shigella* than one might have anticipated. Who used soap in the hand washing study? Was it only the mothers, or was it everyone?

Dr. Khan

It was the mothers who are responsible for washing the bottoms of the children. Moreover, most of the children are fed by the mother. She also washes the hands of those young children who can feed themselves. Thus, she is the crucial person for using soap and water.

Dr. Keusch

The study shows the importance of the mother as the intermediary in transmitting *Shigella*.

Dr. Khan

We have seen that when the mother is infected, the secondary infection rate is much higher than when the father is infected. It is not only the mother but also the older female child (above the age of 15) who performs the duties of the mother. If a child is able to eat by himself, the instruction was to wash his hands before eating.

Dr. Keusch

However, the question is, "If a child was of the age group where the mother washes his hands, the mother's hand washing should have terminated transmission." If the index case was a child over that age group, then it is quite possible that the child was the transmitter, and hand washing by the mother was irrelevant. Thereby, the age of the index case may be interesting.

Dr. Levine

I have a question for Dr. Aziz or Dr. Mujibur Rahaman. It is about the seasonality of shigellosis in Teknaf, which peaks in June-July, if I remember. Is June-July the wet season? If that be the case, then we have seen from studies in Central America that both the availability and quantities of water are important; and that even impure water can decrease the incidence of shigellosis. The peak of shigellosis in Teknaf, where presumably water was abundant, would suggest that other mechanisms operated, mechanisms which enhanced disease transmission. Would you comment on what is going on?

Dr. Aziz

June and July is the wet season. Even though there is a lot of water we found a peak in disease incidences at that time. Another study in the same area, where we closely monitored the actual amount of water used by individuals, has shown the same thing: that shigellosis is a water-washed disease. People who used more than a certain amount of water had very low incidence rates, and those who used less water had high incidence. In the same areas where there is a lot of water available, still the incidence of shigellosis is high. It does not necessarily follow because more water is available, people will use extra water for personal hygiene and consumption.

We also have found that the *E.coli* (coliform) recovered from tubewell water was high when the water table rose. In other words, there is a possibility that at that time the organisms spread all over, resulting in transmission by water. There are other modes of transmission as well -- by touch or contact. But I do not know what role immunity plays in it. So, the issue is very complex. It may depend on how much water a person is using and what the quality of the water is.

Dr. Levine

What about the breeding of flies, the fly density in the wet season versus in the dry season?

Dr. Aziz

In the wet season the fly density is higher.

Dr. Kostrezewski

Another factor may have contributed to this. It is the availability of less sunlight, which is a very powerful disinfectant. There is less sunlight in the wet season.

Dr. Aziz

I would consider that as a very big factor because, for a number of days, average hours of sunshine is much less during the rainy season, as opposed to the dry season. On the other hand there may be enough sunshine, because the days are longer at this time compared to the dry season.

Dr. Glass

We have data which represent three sets of seasonalities for dysentery, one each for Teknaf, Matlab and Dhaka. The incidence of shigellosis in all these three places is low in the cold, dry season. In Teknaf, the incidences are higher in July/August. In Dhaka, they go up before the monsoon. In Matlab they are highest in July and August. Incidences in all these places are low in February. So you have distinct seasonality. Matlab is a low-lying riverine area, while Teknaf is mostly hilly.

Dr. Keusch

The epidemiological behaviour of an organism is a matter of how the organism adapts to the environment and the host. There are really two different eco-systems we should be thinking about: one is outside the human and the other is inside the human. This relates to the survival of the organism, its presence throughout the year, and what it does once it gets into the host. It is important not to forget about the organism's biological properties that allow it to adapt to environmental changes.

In various parts of the world, seasons can differ from before the rains to during the rains, to after the rains, and with this can differ the behaviour of the same basic disease - causing organisms. Important factors also affecting the behaviour of these organisms are peculiarities of particular societies.

I think we really must begin to understand the qualities of the organism that allow it to survive or not survive in a given environment, if we are to focus on transmission of the disease. For example, peculiarities of *Shigellas* are that they are adapted to humans. There will be no disease unless there exists a large population of primates to be infected. Nobody has presented any data on survival of the organism with a different serotype, in different species or the environment. The only comment I heard was Mike Levine's. But there was no data. Maybe it is true. I would like to see some data. This is obviously something very important. What is the organism and how does the organism survive outside the host? How is it

transmitted between the hosts? Once we understand that, we can focus on why one organism causes more severe disease or more frequent clinical disease or infection. I think we must deal with both the ecosystems.

Dr. Aziz

The well-known problem is, "If you have found *E.coli* three or four logs higher than *Shigella*, there is no medium that really can sort them out". We do not have any medium which selects strongly enough. Thus, it is very difficult or impossible to isolate *Shigella* from the environment. There have been only a few reports of such *Shigella* isolation.

Dr. Levine

There is some amount of data on isolation from the environment, particularly on cultivation of *Shigella sonnei* from bedpans and toilets. In this context is the broad discussion we had this morning, including the part about *sonnei* and its occurrence. Countries have a lot of data on duration and persistence of *sonnei* and the special interventions one must take to eradicate *sonnei* problems.

For example, washing of toilets causes an aerosol, and *sonnei* spread throughout the comode area by a flush mechanism. If one covers and prevents the aerosols, one significantly decreases contamination of the environment.

If one looks at patients with *dysenteriae* 1, *flexneri* and *sonnei*, one finds that you take routine media that are helpful, such as *Salmonella-Shigella* media from a bloody mucoid specimen that has 8 or 9 logs of the mechanism per gram, and try to grow *shiga bacillus*. You get negative results. That tells you that this is a fastidious organism. Even on culture media, it does not survive very well.

As far as I know, *Shigella flexneri* is the only bacterial enteropathogen for which there is clear-cut epidemiologic evidence that it is spread by flies. In many areas, without substantiation, we associate flies with being involved in the transmission of a number of organisms. For *Shigella*, however, there is substantiation, thanks to studies done in the U.S. in the mid-forties when that area was developing.

These studies involved massive disease prevention, using DDT in some areas but not in others. In the DDT areas, there was a decrease in infant mortality, a decrease in *Shigella* incidence. The following year, the researchers switched. They sprayed the control areas of the previous year, but not the previously treated areas. The study clearly established the fact that flies are responsible for the transmission of *Shigella flexneri*.

To some degree, whenever we talk about seasonality and *Shigella flexneri* in developing areas we would have to talk about flies. This confirms the interesting data presented by Dr. Khan, showing that 8 percent of the flies

caught in the Dhaka area grow *Shigella flexneri*. That is positively fascinating; and similar results have been found in at least one other study, done at the American University in Beirut. That study looked at a certain fly, a sub-species of *domestica* in Egypt, a fly that breeds in human faeces and feeds on human foods. In those areas, shigellosis is highly endemic.

Dr. Kostrezewski

I think this is a very good point and I agree with that. There is also enough data in the Russian literature on the importance of flies in spreading *Shigella*.

Dr. Hanna

I think, there is more than one mode of transmission. In different countries, in different seasons, there may be different ways of transmission. I would like to know what is the period of time between infection and illness -- because, as we know, very low infecting rates may have very long infecting results. Perhaps *Shigella* infections can transmit; and, if there is some break in immunity, it may make the illness appear. We had epidemics of *Shigella sonnei* in Warsaw in 1966. I counted the number of patients every day coming to the out-patient clinic. Men from whom I collected the data were all from swamps and there was coincidence with rain. From the Metereological Institute I collected data about rain. There were many more patients on rainy days than on non-rainy days. Thus, I believe that if *Shigella* was not transmitted by water, then it was a question of false data.

LIST OF PARTICIPANTS

OVERSEAS

Dr. Roger A. Feldman
Chief, Enteric Diseases Branch
Bureau of Epidemiology
Center for Disease Control (CDC)
Atlanta, Georgia 30333
U S A

Dr. Samuel B. Formal
Chief, Dept Bacterial Diseases
Department of the Army
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D.C. 20012
U S A

Dr. Gerald T. Keusch
Professor of Medicine
Chief, Division of Geographic Medicine
Tufts University of Medicine
New England Medical Center Hospital
136, Harrison Avenue
Boston, Massachusetts 02111
U S A

Dr. J. Kostrzewski
Polska Akademia Nauk
Palac Kultury I Nauki
Skrytka Pocztowe 24
00-901 Warszawa
Poland

Dr. Myron M. Levine
Director
Center for Vaccine Development
University of
Maryland School of Medicine
29, South Green Street
Baltimore, Maryland 21201
U S A

Dr. Leonardo Mata
Director
Institute De Investigaciones En Salud (INISA)
University De Costa Rica
San Pedro
Costa Rica

Dr. H. Stypulkowska-Misiurewicz
National Institute of Hygiene
Chooimska 24
00-791 Warsaw
Poland

Dr. William P. Reed
Professor of Medicine
University of New Mexico and
Association for Research (151)
VA Medical Center
2100, Ridgecrest Dr. Se
Albuquerque, NM 87108
U S A

Dr. Duan Shu-Cheng
Associate Professor of Pediatrics
Deputy Director of
Children's Hospital
Attached to
Shanghai First Medical College
Shanghai
China

Dr. R. Sutton
Scientist
Control of Diarrhoeal Diseases
World Health Organization
1211 Geneve 27
Switzerland

Dr. Yoshifumi Takeda
Associate Professor of
Bacteriology and Serology
Osaka University
Research Institute for
Microbial Diseases
Yamada-Kami
Suita, Osaka
Japan

LOCAL

Dr. M.M. Rahaman	ICDDR,B
Dr. K.M.S. Aziz	"
Dr. R. Laila Akbar	"
Dr.Wirjawan	"
Dr. K.M.A. Aziz	"
Mr. M.K. Chowdhury	"
Dr. A.S.G. Faruque	"
Dr. Roger I. Glass	"
Dr. Kh. Z. Hasan	"
Mr. K.M. Belayet Hossain	"
Dr. Anwar Hossain	"
Dr. Imdadul Huq	"
Dr. Rafiqul Islam	"
Dr. Iqbal Kabir	"
Dr. Shahjahan Kabir	"
Dr. Asma Khanam	"
Dr. M.U. Khan	"
Dr. G.H. Rabbani	"
Dr. Shafiqul Alam Sarker	"
Dr. Md. Yunus	"
Dr. A.M. Molla	"
Dr. Moshaddeque Hossain	"
Mr. M.A. Wahed	"
Mr. Shafiqul Islam	"
Mr. Sirajul Islam	"
Dr. Subrata Chakma	Divn. Malaria Officer, Comilla
Deputy Civil Surgeon	Cox's Bazar
Dr. Nawab Hossain	Health Officer, Chittagong Municipality