J DIARRHOEAL DIS RES 1997 Jun;15(2):65-70
0253-8768/97 $ 1.00+0.10

Detection of Non-culturable *Shigella dysenteriae* 1 from Artificially Contaminated Volunteers' Fingers Using Fluorescent Antibody and PCR Techniques

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SUMMARY

Epidemiological studies have demonstrated that hands may be an important vehicle for transmission of shigellosis. The present study was carried out to find out the survival potential of *Shigella dysenteriae* 1 on fingers of volunteers. Finger surface was inoculated with 10^5 cfu of *S. dysenteriae* 1 and then the bacteria were detected using conventional culture, PCR and fluorescent antibody (FA) techniques after different time intervals. It was found that *S. dysenteriae* 1 survived for up to one hour in culturable form but up to four hours in non-culturable form on human fingers. The non-culturable *S. dysenteriae* was detected by PCR and FA techniques. This study elaborates on the role that fingers have in the transmission of shigellae.

Key words: Dysentery, Bacillary; *Shigella dysenteriae;* Disease transmission; Polymerase chain reaction; Flourescent antibody technique

INTRODUCTION

Shigellosis is mainly associated with low levels of personal hygiene and sanitation (14). Shigellosis is hyperendemic in some parts of Bangladesh, and occasionally flares into epidemics. In routine admissions in a diarrhoeal disease hospital in Bangladesh, the isolation rate of shigellae is approximately 11 to 12% (7).

Shigellosis is mainly transmitted by the faecal-oral route. Several studies have demonstrated that hands may be an important vehicle for transmission of shigellae (5,15,24). Hardy and Watt (6) isolated shigellae from the fingers of mentally retarded individuals in a custodial institution.

The non-culturable phenomenon, a survival strategy of shigellae has been reported (1, 9). The potential health hazard presented by non-culturable shigella spp. may be significant, because of the possibility of conversion into culturable state in the appropriate milieu. It has recently been shown that non-culturable bacteria can revert to the culturable state (18,21,28).

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However, one difficulty in elucidating the potential hazard of non-culturable pathogenic bacteria is the inability of routine bacteriological methods to detect such germs from environmental samples, specially when they are present in low numbers. Polymerase chain reaction (PCR) can detect low number of germs in a sample. This technique allows a specific segment of DNA to be amplified by a factor of 10^6 or more within hours (22). It depends only on the presence of target DNA. Thus, PCR is potentially able to detect the presence of non-culturable cells.

On the other hand, it has also been reported that fluorescent antibody (FA) technique is a highly selective and sensitive method for detection and identification of bacterial population in the environment (10, 11, 23, 26). Thus, this technique can also be employed for detection of low numbers of germs, including non-culturable shigellae from environmental samples (9).

Even though hands play an important role in the transmission of shigellae, there is little information about the persistence of shigellae on contaminated fingers. Hutchinson, using conventional cultural techniques (8), has

shown that *Shigella sonnei* can survive for up to three hours on fingers in temperate climates, but there is no information on survival of *S. dysenteriae* 1 in a tropical climate. Moreover, when the study on *S. sonnei* was conducted there was no knowledge about non-culturable organisms which could potentially transmit the disease. We are also not aware of any study about the survival of *S. dysenteriae* 1 on fingers using culture, PCR and FA. Therefore, the present study was undertaken to find out the persistence of *S. dysenteriae* 1 on human fingers using culture, FA and PCR techniques.

SUBJECTS AND METHODS

Volunteers and bacterial strain

Four male, healthy volunteers were selected for this study. One clinical strain of *S. dysenteriae* 1 was obtained from the Microbiology Laboratory of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). This strain was reconfirmed by morphological, cultural, biochemical, and serological tests following standard procedures (13).

Preparation of inoculum

The strain was first inoculated onto MacConkey agar (MA) and incubated at 37 °C for 24 hours. A loopful of growth was suspended in 10 mL phosphate buffered saline (PBS, pH 7.3). The 90% transmittance of the suspension at A_{585} was measured with a spectrophotometer (Coleman Junior, IIA model 6/20A: Perkin Elmer corp.). Bacterial counts were assessed following standard procedures.

Inoculation of fingers

The finger surface of volunteers was swabbed with 70% alcohol and allowed to dry following washing with sterile distilled water. A drop of 20 µl inoculum (~10^5 cfu) was placed onto each finger tip of the left hand, except the thumb, and was spread over an area of 2 cm x 1 cm. The right hand fingers were not inoculated and used as negative control. The volunteers did not touch anything and were confined to the laboratory during the experiment.

Sampling

Each inoculated finger was washed once in 2 mL washing solution (0.25 Ringer solution containing 0.1% Tween 20) by rubbing with the thumb of the same hand. The thumb was then sterilised by alcohol and rinsed with sterile distilled water. Different fingers were washed at different time intervals (0, 5, 10, 20, 30, 60 minutes, 2, 3, 4, 5, and 8 hours), and shigellae were counted from the Ringer solution. Aliquots of the solution were stored at –20 °C for detection of shigellae by PCR and FA technique.

Culturable cell count

The viable germ numbers in the finger washes were enumerated on MacConkey agar (MA), Salmonella-Shigella agar (SS), xylose lysine deoxycholate agar (XLD), and Hektoen enteric agar (HEA) following standard procedure with a sensitivity of 1 cfu/cm^2 (9). Two to three colonies from the plates were tested with *S. dysenteriae* 1 antiserum (Difco) by slide agglutination for confirmation of the strain. The bacterial count was expressed as cfu/cm^2 of finger surface. When the count of culturable cells came down to less than 1 cfu/cm^2, then the culturability of the germs was tested by culturing on MA following enrichment with Gram-negative (GN) broth.

DNA extraction

When there were no culturable germs, one mL sample was put in an Eppendorf microfuge (model 5415C) and centrifuged at 3000 x G for three minutes to remove skin particles and debris. The supernatant was collected and DNA of bacterial cells was extracted by the method described by Islam *et al.* (9). The extracted DNA was stored at –20 °C if the subsequent experiment was not carried out immediately. The DNA was extracted from the non-culturable form of *S. dysenteriae* after 2, 3, 4, 5, and 8 hours of exposure on the fingers.

DNA amplification by PCR

The amplification of *ipa*H sequences was performed in 100 µl reaction mixture in polypropylene microfuge tubes (Perkin Elmer Cetus). The reaction mixture was prepared using 10 µl buffer solution, 2 µl of each deoxy-ribonucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 0.5 µl of Taq DNA polymerase, 1 µl of each primers (H8, 5'-GTTCCTTGACCGCCTTTCCGATAC-3' and H15, 5'-GCCGGTCAGCCACCCTC-3') which were derived from an invasion plasmid of *S. flexneri* M90T, (27) 8 µl of extracted DNA and sterile deionized water to a final volume of 100 µl. The reaction mixture was overlaid with 50 µl mineral oil. The PCR reaction was done for 35 cycles of one minute each at 94 °C (for denaturation), 1.5 minute each at 60 °C (for annealing of primers to single-stranded DNA), and 0.25 to one minute each at 72 °C (for DNA polymerase-mediated extension) according to the procedure described by Echeverria *et al.* (3).

Analysis of PCR product

The amplified DNA was analysed by agarose gel electrophoresis. The PCR product and molecular weight marker (HaeIII digest of ϕX174 replicative form DNA) were subjected to electrophoresis. The separated product was visualised by UV transilluminator (Ultraviolet products

Inc. San Gabriel, California, USA) after staining with ethidium bromide.

Hybridisation of PCR product

The PCR product was also identified by hybridisation technique according to the ECL (enhanced chemiluminescence) direct nucleic acid labelling and detection systems protocol (Amersham, UK). After electrophoresis, the gel was processed with the denaturation solution (1.5 M NaCl, 0.5 M NaOH) and neutralisation solution (1.5 M NaCl, 0.5 M Tris HCl, pH 7.5), followed by washing with deionised water. The separated product was then transferred to a nylon membrane by capillary blotting technique (17). A single-stranded *ipa*H probe, described previously (27) was labelled with horseradish peroxidase (20). The hybridisation between PCR product fixed on the blot and the labelled probe was carried out under stringent conditions. The blot was removed immediately from the hybridisation medium and washed twice in primary wash buffer (0.4% SDS, 0.5 x SSC) for 10 minutes at 55 °C followed by another wash in secondary wash buffer (2 x SSC) for five minutes at room temperature. The occurrence of hybridisation was detected using ECL detection reagents (Amersham, UK). Autoradiography film was used for detecting hybridised product on the nylon membrane.

Fluorescent microscopy

One mL of hand wash sample was put into an Eppendorf microfuge tube and centrifuged at 3000 x G for three minutes to remove the skin particles and debris. Supernatant was collected and was centrifuged at 15,000 x G for five minutes. The supernatant was discarded and the pellet was resuspended in 25 µl of PBS (pH 7.3). 5 µl of this suspension was applied to a microscopic slide. The germs were stained with *S. dysenteriae* 1 polyclonal antiserum (Wellcome Diagnostics, Dartford, United Kingdom) and fluorescein isothiocyanate-conjugated anti-rabbit goat serum. The slide was mounted under a coverslip with buffered glycerol (pH 8.3) and then examined under an epifluorescence microscope (model BH-2; Olympus). *S. dysenteriae* 1 was used as a positive control.

Data analysis

The experiment was carried out with four volunteers and was repeated four times with each individual. The count of the culturable *S. dysenteriae* 1 on the fingers was calculated at intervals of 0, 5, 10, 20, 30, 60 and 120 minutes. Firstly the average counts were obtained from the 4 readings of each individual; then the mean values of the counts were expressed with standard deviation (SD) from the average counts of the four individuals at each sampling interval.

RESULTS

Survival of *S. dysenteriae* 1 in the culturable state on the finger was considered as the ability of that organism to multiply and to form colonies on various selective media, e.g. MA, SS, XLD, and HEA. Fig. 1 shows the survival pattern of *S. dysenteriae* 1 on the fingers of four volunteers. The result demonstrated that the growth response of *S. dysenteriae* 1 was similar on all the media used. The initial count was over 10^5 cfu/cm^2 which gradually decreased with time and the cells were not culturable after 60 minutes on the fingers of all the four individuals.

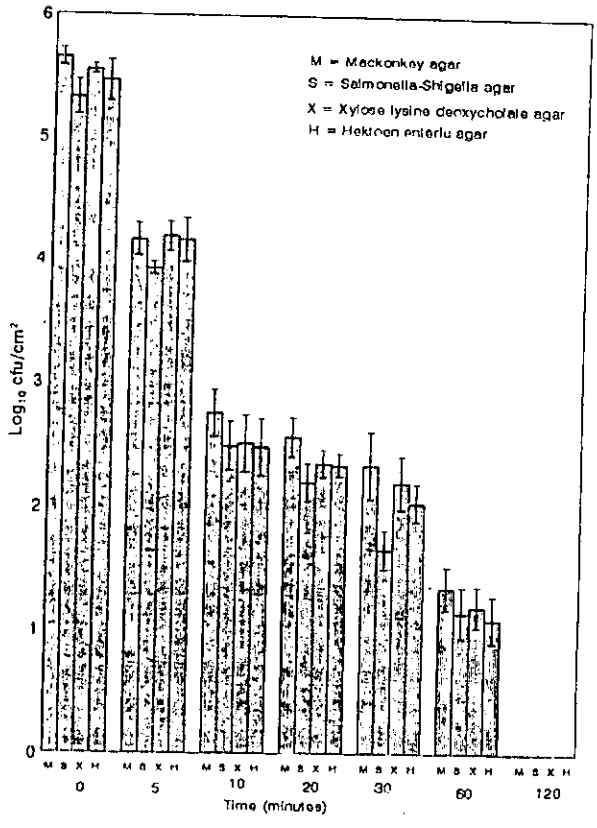

Fig. 1: Culturability of *S. dysenteriae* 1 on different media after inoculation on volunteers' fingers. Log_{10} counts of *S. dysenteriae* type 1 on fingers ar different time intervals are shown with ± 1SD. Symbols: MacConkey agar (M), Salmonella-Shigella agar (S), xylose lysine deoxycholate agar (X) and Hektoen enteric agar (H)

The DNA was subjected to amplification by PCR and the PCR products were analysed by agarose gel electrophoresis (Fig. 2A). The analysed PCR products were then transferred to nylon membrane and were hybridised with the *ipa*H probe. Figure 2A shows that the finger washing samples collected at 2, 3, and 4 hours intervals generated the expected 700-bp fragment of the H locus which

hybridised to the *ipa*H probe (Fig. 2B), indicating the presence of non-culturable *S. dysenteriae* 1 on the fingers for up to four hours.

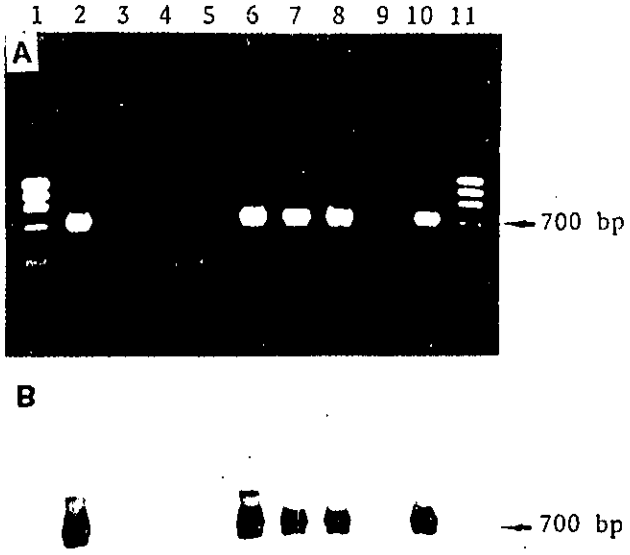

Fig. 2: Analysis and hybridisation of PCR products : (A) The extracted DNA from contaminated finger washings was subjected to specific amplification of invasion plasmid antigen H locus (*ipa*H) DNA by PCR. The PCR products were then analysed by 0.8% agarose gel electrophoresis. Lanes : 1 and 11, Hae III digest of ϕX174 replicative-form DNA as markers; 2 and 10, positive controls with DNA from culturable *S. dysenteriae* 1; 3 and 9 negative controls without DNA; 4 and 5, finger washing samples after 8 and 6 hours respectively which did not generate any bands; and 6, 7 and 8, finger washing samples after 4, 3 and 2 hours respectively which generated bands indicating the presence of non-culturable *S. dysenteriae* 1 (B) Hybridisation of the corresponding DNA products with horseradish peroxidase-labelled *ipa*H probe on nylon membrane.

The non-culturable *S. dysenteriae* (Fig. 3A) on the fingers were also detected up to four hours using FA technique and appeared as rounded bodies rather than rods, contrasting with the culturable form of *S. dysenteriae* 1 (Fig. 3B).

DISCUSSION

In the present study, *S. dysenteriae* 1 was shown to survive in a culturable state for up to one hour but persisted for up to four hours as non-culturable state on volunteers' fingers. The non-culturable stage in the life cycle of a bacterium appears to be a strategy for survival when the organism is exposed to conditions that are less than optimal for cell growth and division.

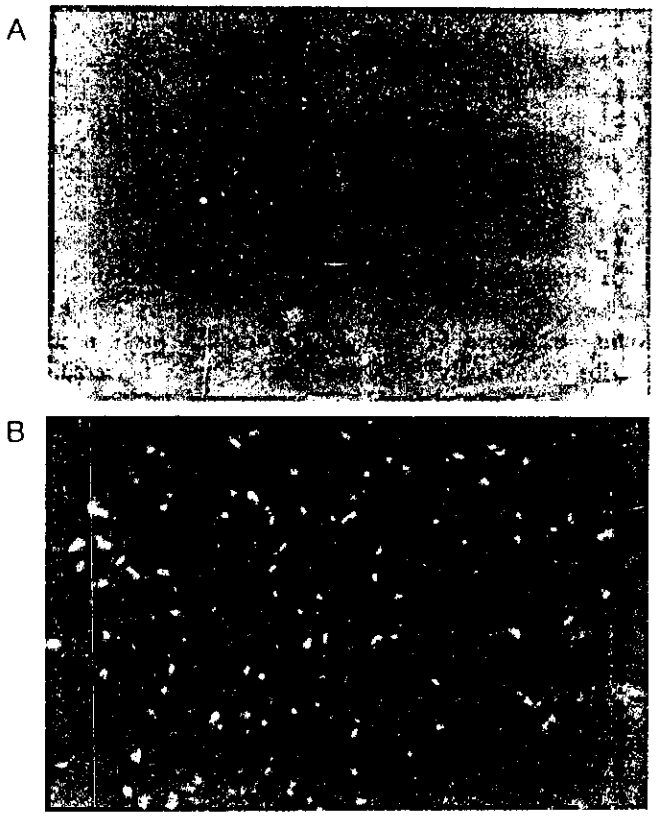

Fig. 3: Fluorescence photomicrograph of *S. dysenteriae* 1. (A) non-culturable *S. dysenteriae* 1 obtained from the finger washings of volunteers (B) culturable *S. dysenteriea* 1 as a positive control (magnification x 1000 for both)

There is evidence that *S. sonnei* can survive on human hands (8), and human hands have been implicated as sources of transmission in outbreaks of shigellosis (15). This has led us to explore the persistence of *S. dysenteriae* on human fingers. The non-culturable form of *S. dysenteriae* has been investigated, because this non-culturable form has been shown to occur in *V. cholerae*, *Salmonella enteritidis*, enteropathogenic *E. coli* and other pathogens (1, 21,29). It is, therefore, important that methods to detect such germs be developed. Moreover, a technique to detect small numbers of cells is essential, since many bacteria are present in the environment only at low densities. Such detection is especially important for bacteria, such as *Shigellae*, which can produce disease after ingestion of as few as 10 organisms (16). The PCR technique seems ideally suited for this goal, since it potentially allows amplification of the DNA obtained from only a few germs (25).

In the present study, the persistence of *S. dysenteriae* on volunteers' fingers has also been observed by fluorescence microscopy for up to four hours, despite the fact that after one hour the cells could not be cultured. This study shows that *S. dysenteriae* became non-culturable within one hour on finger surface, which may be due to dryness and the low pH of the skin (19). Moreover, antimicrobial substances (e.g. lysozyme, complex lipid products, antibodies, primarily IgA and IgG) excreted from the skin (12) may play a significant role in the quick transformation of culturable to non-culturable state of *S. dysenteriae* on human fingers. However, these non-culturable bacteria, if still viable, may revert to the culturable form in a favourable environment. Colwell *et al.* (2) observed that non-culturable *V. cholerae*, when ingested by volunteers, reverted to culturable form and were excreted in the stools.

The present study shows that the present methods of detecting shigellae by conventional culture techniques are not fully adequate. This is corroborated by outbreaks of shigellosis in which no organisms can be isolated from the suspected transmission vehicles by conventional culture techniques (8). However, the PCR and fluorescent antibody methods allow the detection of these non-culturable *S. dysenteriae* germs, even if present in very low numbers.

The non-culturable state reported here for *S. dysenteriae* 1 may be significant for understanding the epidemiology of shigellosis. Recently, a community-based study in an endemic area of Thailand has demonstrated that non-culturable shigellae detected by PCR can cause dysentery with similar clinical characteristics as when caused by shigellae (4). Therefore, it is possible that such non-culturable shigellae may pose health problems. The results of this study, therefore, demonstrate the significance of the persistence of non-culturable *S. dysenteriae* 1 on fingers, which may be important from a public health point of view.

ACKNOWLEDGEMENTS

This research was supported by Swiss Development Co-operation (SDC) and the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). The ICDDR,B is supported by countries and agencies which share its concern for the health problems of developing countries. Current donors include: the aid agencies of the Governments of Australia, Bangladesh, Belgium, Canada, China, Denmark, Germany, Japan, The Netherlands, Norway, Republic of Korea, Saudi Arabia, Sweden, Switzerland, the United Kingdom, and the United States; international organisations, including Arab Gulf Fund, Asian Development Bank, European Union, International Atomic Energy Centre, United Nations Children's Fund (UNICEF), United Nations Development Program (UNDP), United Nations Population Fund (UNFPA) and World Health Organization (WHO); private foundations, including Child Health Foundation, Ford Foundation, Population Council, Rockefeller Foundation and Sasakawa Foundation; and private organisations, including American Express Bank, Bayer A.G., CARE, Family Health International, Helen Keller International, Johns Hopkins University, Procter Gamble, RAND Corporation, SANDOZ, Swiss Red Cross, University of California Davis, and others.

We thank Mr. Md. Anisur Rahman for typing the manuscript.

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