Date 7 Dec 1983

8/12/83

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Elisabeth Carniel

Trainee Investigator (if any) _____

Application No. 83-044

Supporting Agency (if Non-ICDDR,B) _____

Title of Study Role of Yersinia infection in childhood diarrhoea and pseudo-appendicular syndrome in Bangladesh

Project status:
- (x) New Study
- ( ) Continuation with change
- ( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

Source of Population:
- (a) Ill subjects (Yes) No
- (b) Non-ill subjects Yes (No)
- (c) Minors or persons under guardianship (Yes) No

Does the study involve:
- (a) Physical risks to the subjects Yes (No)
- (b) Social Risks Yes (No)
- (c) Psychological risks to subjects Yes (No)
- (d) Discomfort to subjects Yes (No)
- (e) Invasion of privacy Yes (No)
- (f) Disclosure of information damaging to subject or others Yes (No)

Does the study involve:
- (a) Use of records, (hospital, medical, death, birth or other) (Yes) No
- (b) Use of fetal tissue or abortus Yes (No)
- (c) Use of organs or body fluids (Yes) No

Are subjects clearly informed about:
- (a) Nature and purposes of study Yes (No)
- (b) Procedures to be followed including alternatives used Yes (No)
- (c) Physical risks Yes (No)
- (d) Sensitive questions Yes (No)
- (e) Benefits to be derived Yes (No)
- (f) Right to refuse to participate or to withdraw from study N.A Yes No
- (g) Confidential handling of data (Yes) No
- (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure N.A Yes No

5. Will signed consent form be required:
   - (a) From subjects Yes (No)
   - (b) From parent or guardian (if subjects are minors) Yes (No)
6. Will precautions be taken to protect anonymity of subjects (Yes) No
7. Check documents being submitted herewith to Committee:
   - ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies)
   - V Protocol (Required)
   - V Abstract Summary (Required)
   - NA Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   - NA Informed consent form for subjects
   - NA Informed consent form for parent or guardian
   - NA Procedure for maintaining confidentiality
   - NA Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

I agree to obtain approval of the Ethical Review Committee for any changes involving the rights and welfare of subjects before making such change.

Principal Investigator

Trainee

SECTION I - RESEARCH PROGRAMME

1. Title: Role of Yersinia infection in childhood diarrhoea and pseudo-appendicular syndrome in Bangladesh

2. Principal Investigator: Elisabeth Carniel

   Co-Investigator: Thomas C. Butler and Nur Haque Alam

3. Starting date: January 1, 1984

4. Completion date: October 1, 1984

5. Total direct costs: $20,184

6. Scientific Programme Head: Thomas C. Butler, M.D.

This protocol has been approved by the Pathogenesis-Therapy Working Group.

Signature of the Programme Head: T Butler

Date: 7-12-83

7. Abstract:

The dramatic rise in the incidence of yersiniosis in recent years is a result of a worldwide extension. A previous study in Bangladesh failed to find Yersinia. Afterwards four Yersiniae (1 Yersinia enterocolitica and 3 Yersinia intermedia) has been isolated in post-mortem studies from blood, spleen, bowel content, lungs. The present study will try to investigate the frequency of yersiniosis exploring invasive diarrhoea (in children less than 7 years old), inflamed lymph-nodes, post-mortem cases and pig's tongues. To enhance isolation

a new culture method employing a potassium hydroxide treatment and selective media for Yersinia will be combined. The virulent or avirulent strains will be differentiated by using Congo red agar and will be sent for biotyping, serotyping and phage typing to the "centre de reference des Yersinia" Institut Pasteur de Paris, Professor H.H. Mollaret.

8. Reviews:

a. Ethical Review Committee : _____

b. Research Review Committee: _____

c. Director: _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objectives:

Using new culture method employing potassium hydroxide treatment and new selective media, this study attempt to achieve two objectives:

a. to determine the frequency of Yersinia infection associated with different clinical syndromes (invasive diarrhoea, pseudo-appendicular syndrome, fatal diarrhoeal cases).

b. to investigate if, as in other countries, pigs are potential reservoirs for these bacteria.

2. Background:

Three species in the genus Yersinia are important in human and animal pathology: Yersinia pestis, Yersinia enterocolitica and Yersinia pseudotuberculosis.

The major clinical feature due to Y. enterocolitica is an acute enteritis. After 4 to 10 days of incubation, diarrhoea of variable intensity sometimes bloody occurs. A majority of patients presents with fever, generally low grade and abdominal pain. A minority also complain of nausea and rarely vomiting. Dehydration is a rare complication. Ileitis can mimic an appendicitis and lead to an appendicectomy. Y. enterocolitica enteritis is largly prominent among children (<7 years). Secondary manifestations can also occur: aseptic reactional oligoarthritis which are seen with a significantly high frequency in patients

with $HLAB_{27}$ and erythema nodosum, especially in adult female. Lastly, severe forms are observed in patients with underlying conditions: cirrhosis, diabetes, hemochromatosis, thalassemic children, wasting or undernourished individuals and immunocompromised hosts. They are due to the passage of the bacillus in the systemic circulation and to its peritoneal, hepatic or osteo-articular localization which leads sometimes to septicemia. Y. pseudotuberculosis is also responsible for a pseudo-appendicular syndrome due to a mesenteric lymphadenitis.

The Yersiniae are gram negative coccobacilli, non-capsulated, non-sporulated, facultative aero-anaerobic rods, growing on usual media and motile only below 29°C with peritrichous flagella. They ferment glucose, reduce nitrates to nitrites. They are catalase (+), oxidase(-). They belong to the enterobacteriaceae. Specific characteristics are: 29°C as the optimal temperature of growth, fast urease, ONPG positive only below 30°C. Y. pseudotuberculosis differs from Y. enterocolitica by the fermentation of rhamnose, the non fermentation of cellobiose and saccharose and the lack of ODC.

Because Yersinia grows more slowly than non-yersinia organisms, its population is quickly overgrown and easily masked when streaked on a weakly selective isolatory agar. New culture methods for Yersiniae have been discovered; they highly increase the rate of isolates:

1. a new culture method employing a potassium hydroxide treatment was compared with the conventional cold enrichment method for efficacy in recovering yersinia species from naturally and artificially contaminated food. The new method increased the

yield of yersinia sp. four-fold and the sensitivity 100-fold, shortened the incubation period and appreciably decreased the growth of non-yersinia bacteria.

2. Comparing selective media for recovery of Y. enterocolitica, Head et al found CIN agar by far the most effective medium. This medium was highly selective and almost completely inhibited the fecal flora while supporting luxuriant growth of Y. enterocolitica. With 10^1 colony forming units, CIN yielded a 100% recovery of the test strains.

There is no standard method for isolation of yersinia but the combination of these two technics which is used by the "laboratoire de la ville de Paris" seems to significantly increase the sensitivity of isolation.

The phenotype of Y. enterocolitica is based upon three parameters:

1. The biovar. Five biovars of Y. enterocolitica sensu stricto are described following Wauter's classification on the basis of the production of a lipase, indol, fermentation of xylose, trehalose and saccharose. Recently, strains ressembling Y. enterocolitica biovar 1 have been separated from this classification and described as new species: Yersina intermedia (Rhamnose (+), ∝ Methyl D glucoside(+), Melibiose (+), Raffinose (+)), Y. fredericksenii (Rhamnose (+) but melibiose (-)) and Y. Kristensenii ((Sucrose (-), V.P (-)).
2. The serovar, defined by the O antigens determinants. More than 50 serovars are described at the present time.
3. The phage type which is based upon the study of the lytic action either of bacteriophages from lysogenic strains of Y. enterocolitica and of bacteriophages isolated from sewage water.

These three parameters are significantly linked in each phenotype of Y. enterocolitica.

European countries have a high incidence of yersiniosis. The incidence of Y. enterocolitica among the causative agents of diarrhoea is nearly as high as Salmonella, Shigella and Campylobacter in Norway, Sweden, Hungary and Western Germany. The strains isolated in human pathology in Europe belong essentially to the biovar 4, serovar 3, phage type VIII (4/3/VIII) and more rarely to the phenotype $2/9/X_3$. In the Americas, the number of isolates is higher in the North; in Canada ($4/3/IX_b$) and in the United States (1/8/?). Y. enterocolitica has also been described in southern America (Brazil, Peru, Guatemala and Chile with the prevalence of the phenotype 4/3/VIII); in Africa (South Africa ($4/3/IX_a$), Zaire (4/3/VIII), Marocco (4/3/VIII) and Nigeria); in the near East countries (Israel (4/3/VIII), Egypt (3/3/XI) and Iran ($4/1,2_a,3/XI$)) and in Australia. In Asia Y. enterocolitica has often been isolated in Japan (4/3/VIII). In India the phenotype $2/9.16/X_2$ has been found in 3 human strains and the phenotype $2/9.16/X_3$ from one human sample, one pig and one cat. In Bangladesh, a recent study by Samadi et al in Dhaka failed to find Yersinia. They examined 325 stool samples from human, 190 stool samples from animal with diarrhoea and 30 human appendices. No Y. enterocolitica or Y. enterolocitica "like" was detected. The authors concluded that the failure to find isolates of Y. enterotocolitica may be due to Dhaka's warm climate and to the virtual absence of pig rearing in Bangladesh. Nevertheless, Butler et al have recently isolated from tissues at post-mortem examination four Yersiniae: one Y. enterotocolitica serotype O:7,8 from small bowel

contents and spleen, one Y. intermedia from blood, one Y. intermedia from a mesenteric lymph node and one Y. intermedia from

These authors concluded that even if the pathogenicity of Y. intermedia has not yet been firmly established, those cases suggest that these organisms could be either co-pathogens or secondary invaders in the setting of estabilised infection. They add that a further work would be useful to determine the importance of Yersinia infection in tropical diarrhoeal disease syndrome.

With regard to the pathogenicity, the yersiniosis are divided in two groups: the first includes Yersiniae which are adapted to a specific host and which are pathogen for it.

- Yersinia enterocolitica biovar 5 in hares
- " " " 4 in men and pigs
- " " " 3 in chinchillas
- " " " 2 in men and pigs

The second group is composed of Y. enterocolitica biovar 1, Y. intermedia Y. kristensenii and Y. fredericksenii. They are abundantly found in the environmental samples and they are considered as non-pathogenic for the human being. In fact the biovar 1 of Y. enterocolitica, which is not often isolated in human illness in Europe seems to be the main causative agent of diarrhoeal diseases due to Yersinia in the United States. Moreover, blood culture during septicemia in Europe have been positive for Y. enterocolitica biovar 1 or Y. intermedia. Y. fredericksenii has also been isolated from man with unusual syndrome (abcesses, etc...).

3. Rationale

The dramatic rise in the incidence of yersiniosis in recent years is the result of a worldwide extension, an increasing interest of bacteriologists in systematically tracking down the species, and a great improvement of the culture methods. In Bangladesh, four Yersiniae have been isolated from post-mortem studies. This proves the existence of these bacteria here and could explain the origin of some diarrhoeal diseases in which no causative is found. A further work is necessary to determine the importance of Yersinia infection in Bangladesh.

B. SPECIFIC AIMS:

1. To investigate if Yersinia is associated with diarrhoeal diseases in Bangladesh.
2. To see if those bacteria can cause pseudo-appendicular syndromes.
3. To go on exploring post-mortem cases
4. To seek to find the potential reservoir of these organisms exploring pig's tongues.

C. METHODS AND PROCEDURES:

The present study will try to take advantage of the results obtained in Bangladesh and in other countries to enhance isolation of Yersiniae.

The following sources will be investigated:

a. Human samples:

1. Stool - stool samples from patients hospitalized in the general ward or treatment center with the following clinical features: Children less than 7 years old with fever $\geq 37^{\circ}5C$ and diarrhoea.

2. Lymph nodes - collected in collaboration with Dr. Habibur Rahman (holy Family Hospital) and Dr. Altaf(Aragya Nikaton). During laparatomy cases for appendicites or other acute abdominal pain, the appendices and the inflamed peri-appendicular lymph-nodes will be taken and sent to the ICDDR,B for microbial study.

3. Systematic post-mortem study - Yersinia will be systematically strained in bowel content, blood, spleen, liver and mesenteric lymph nodes.

When Yersiniae are found in stools, appendices or lymph nodes, the hospital records will be used to identify the clinical, chemical and bacteriological feature.

b. Pig's tongues:

Each time a pig will be slaughtered in Doyargout the tongue will be collected and studied for the presence of Yersinia.

Bacteriological isolation and identification procedures:

The samples collected in the field will be placed in sterile bags and packed in wet ice during transport.

The procedure for stools and pig tongues will be:

a. direct plating on -

- Mac Conkey agar after KOH treatment
- Oxoid (cefsulodin 7,5 mg/l, Irgasan 2 mg/l, Novobiocin 1,25 mg/l) after KOH treatment
- Difco (Cefsulodin: 4 mg/l, Irgasan .4 mg/l, Novobiocin 2,5 mg/l)

b. an aliquot will be added to phosphate buffer saline double concentrated (PBS) + Modified rappaport medium without carbenicillin (MRB) stored for 24 hours at 30°C and then plated on ;

- Mac Conkey agar after KOH treatment
- Oxoid after KOH treatment

c. another aliquot added to PBS+MRB will be stored for 3 weeks at 4°C and then plated on -

- Mac Conkey agar after KOH treatment
- Oxoid after KOH treatment

For post-mortem cases, appendices and lymph nodes the procedure will be: a direct plating on

- Mac Conkey agar
- Oxoid

b an aliquot will be added to PBS+MRB, stored for 24 hours at 30°C and then plated on

- Mac Conkey agar
- Oxoid

c another aliquot added to PSB+MRB will be stored for 3 weeks at 4°C and then plated on

- Mac Conkey agar

All these media will be incubated at 30°C for 24 to 48 hours.

Identification

On mac Conkey agar, Yersiniae strains are transparent and can be as large as 3 mm in diameter. On Difco and oxoid media the yersinia strains produce a localized pH drop around the colony which, followed by absorption of the neutral red, imparts a red color to the colony. Due to the localized pH drop, a zone of precipitated bile may also be present. Each suspect colony on those media is screened by inoculating a Kligler-Hajna medium and a mannitol motility medium for 24 hours at 25-30°C and a motility medium for 24 hours at 35°C. Y. enterocolitica and Y. pseudotuberculosis colonies on this medium are glucose (+), H_2S (-) with no gas, mannitol (+), nitrate (+), motile at 25°C but not at 35°C. Isolates resembling Y. enterocolitica or Y. pseudotuberculosis in the screening media are listed with the Api 20 gallery.

The differentiation between virulent and avirulent strains of Y. enterocolitica is carried out by using congo red agar. On this medium virulent colonies (CR^+) become dark violet after 48H at 25°C whereas CR^- colonies (avirulent) remain colorless and translucent. All CR^+ strains harbor plasmids between 40 and 50 Mdal.

The strains are then sent for confirmation, biotyping, serotyping and phage-typing to the "centre de reference des yersinia", Institut Pasteur de Paris, P^RH.H. Mollaret with whom there is a collaborative arrangement.

D. SIGNIFICANCE

This study will give us a better idea of the role played by Y. enterocolitica and Y. pseudotuberculosis in diarrhoeal diseases and in pseudo-appendicular syndromes in Bangladesh. It could partly explain long duration diarrhoeal diseases in which no causative agent is found or diarrhoea not responding to ampicillin. The survey of the porks, if positive, will identify a potential reservoir of Yersinia.

E. FACILITIES REQUIRED

1. No new office space is needed
2. Personnel - 1 laboratory technician full time
3. A new laboratory space is needed
4. Hospital support - stools samples will be collected from the ICDDR,B patients. No special care is needed.
5. Bacteriological support - the results of routine cultures for the inpatients will be recorded
6. Logistical support - None
7. Animal support - None
8. Major item of equipment - 1 incubator and 1 refrigerator
9. Other special requirements : selective media for Yersiniae

F. COLLABORATIVE ARRANGEMENTS

There is a collaborative arrangement with the "Institut Pasteur de Paris," service d'ecologie bacterienne, centre national de reference des yersiniae, Professor H.H. Mollaret.

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12. D.A. Schiemann. Isolation of toxigenic Yersinia enterocolitica from retail pork products. 1980. Journ. food. Protect. 43:360-365

ABSTRACT FOR THE ERC

1. Stool samples will be collected in children less than 7 years old with diarrhoeal diseases because Yersiniosis is essentially a childhood infection. Appendices will be collected in all the cases of appendicectomy and inflamed lymphnodes will be collected at the same time.

2. There are no physical, psychological, social or legal risks for these patients.

3. Not applicable.

4. Study numbers will be used for stool samples, appendices and post-mortem cases instead of patient's name.

5. Not applicable

6. No interview will be required

7. This study will give us a better idea of the importance of Yersinia infection in Bangladesh. An appropriate treatment could be carried out if the stool culture is positive for Yersinia

8. When Yersinia is found from stools, appendices or lymph nodes the hospital records will be needed. Samples from post-mortem cases will be collected (lymph nodes, bowel content, spleen, liver...).

SECTION III - BUDGET
(A. DETAILED BUDGET)

				Requirements of Project	
1.	PERSONNEL SERVICES:				
	Name	Designation	Duration	Taka	Dollars
	E. Carniel	Pr. Investigator	1 yr	-	-
	T. Butler	Consultant	1 yr	-	3,750
	Not named	Co-Investigator	1 yr	10,000	-
	Lab.Technician		1 yr	40,000	-
2.	SUPPLIES & MATERIALS:				
	Stool cultures	8000 X 16		128,000	-
	Appendices cultures	1000 X 16		16,000	-
	Pig-tongues cultures	100 x 16		1,600	-
	Oxoid yersinia selective agar			-	875
	Supplement oxoid			-	930
	Difco-Bacto yersinia supplement			-	310
	Difco-Bacto yersinia agar			-	300
	Stool container - glass	500		2,000	
3.	EQUIPMENT:				
	One Refrigerator			-	1,453.50
	One Incubator				800.00
4.	PATIENT HOSPITALIZATION - None				
5.	OUTPATIENT CARE - None				
			Sub Total :	197,600	8,418.50

Detailed budget - page 2	Taka	Dollar
Sub Total :	207,600	8,318.50
6. ICDDR,B TRANSPORT:	40,000	-
7. TRAVEL & TRANSPORTATION	—	—
8. TRANSPORTATION OF THINGS	—	200
9. RENT, COMMUNICATION, UTILITIES - None		
10. PRINTING & PUBLICATION	—	300
11. OTHER CONTRACTUAL SERVICES	10,000	-
Abbattoir service for pig's tongues		
12. CONSTRUCTION, RENOVATION, ALTERNATION - None		
Grand Total :	257,600	8,868,50

B. BUDGET SUMMARY

		Dollars
1.	Personnel	5,833
2.	Supplies	6,150
3.	Equipment	8,254
4.	Hospitalization	-
5.	Outpatient care	-
6.	ICDDR,B Transport	1,666
7.	Travel, persons	-
8.	Transport of things	200
9.	Rent/Communication	-
10.	Printing/Xerox	300
11.	Contractual service	417
12.	Construction	-
	Total :	16,820
	20% over head :	3,364
	Grand Total :	20,184

(Conversion rate US$1 = Tk.24)