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Attachment 1.
(FACE SHEET)

Date 06-05-91

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Dr. Md. Sirajul Islam

Principal Investigator: Dr. M. John Albert Trainee Investigator (if any) _____

Application No. 91-005 Supporting Agency (if Non-ICDDR,B) _____

Title of Study: Investigation of the carrier state and the role of animate and ~~inanimate objects as reservoirs of~~ secondary hosts of shigellae

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).

1. Source of Population:
   (a) Ill subjects (Yes) No
   (b) Non-ill subjects (Yes) No
   (c) Minors or persons under guardianship (Yes) No
2. 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)
3. 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)
4. Are subjects clearly informed about:
   (a) Nature and purposes of study (Yes) No
   (b) Procedures to be followed including alternatives used NA 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 (Yes) No
   (g) Confidential handling of data (Yes) No
   (h) Compensation &/or treatment where there are risks or privacy is involved in NA any particular procedure 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).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ 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)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ 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.

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

M.S. Islam M. John Albert
Principal Investigator Trainee

APPLICATION FOR PROJECT GRANT

1. PRINCIPAL INVESTIGATORS : Dr. Md. Sirajul Islam
Dr. M. John Albert

COINVESTIGATORS : Dr. A.H.Baqui
Mr. Albert Felsenstein
A Senior Medical Officer

ADVISORS : Dr. D. Mahalanabis
Professor R. B. Sack

2. TITLE OF PROJECT : Investigation of the carrier state and the role of animate* and inanimate** objects as reservoirs or secondary hosts of shigellae

*Animate objects:
hand and breast

**Inanimate objects:
cloth, plate, spoon, feeding bottle, water-pot, toy and door-handle

3. STARTING DATE : July, 1991

4. DATE OF COMPLETION : 2 years after starting date

5. TOTAL BUDGET REQUIRED : US$ 118,269

6. FUNDING SOURCE :

7. HEAD OF PROGRAMME : Dr. M. Moyenul Islam
Acting Head
Laboratory Sciences Division

8. AIMS OF PROJECT

a) General aim

To investigate the duration of convalescent excretion of shigellae by humans and the potential of various animate and inanimate objects in the home environment as reservoirs of infection by using both conventional technique and polymerase chain reaction (PCR).

b) Specific aims

i) To find out if a carrier state exists for shigellae after treatment of shigellosis patients with nalidixic acid, pivmecillinum and ciprofloxacin, and if so, to study the duration of faecal excretion of shigellae by using both conventional culture techniques and PCR.

ii) To find out the carriers in families without any disease - community study.

iii) To study the contamination of animate and inanimate objects, water for domestic use and food, with shigellae by using both conventional culture technique and PCR.

c) Significance

By re-examining the carrier status of shigellae by human hosts using very sensitive PCR technique, a more accurate estimate of the duration of excretion following infection with *Shigella* will be obtained. Furthermore, identification of reservoirs for shigellae in inanimate and animate objects will provide invaluable information about the epidemiology of shigellosis. The study may, therefore, influence future *Shigella* control programmes.

9. ETHICAL IMPLICATIONS

Stool specimens or rectal swabs, hand-washings and swabs from clothes will be collected from all individuals enrolled in the study. In addition, washing of the nipple and the surrounding area of the breast will be collected from breast-feeding mothers. The importance of obtaining these specimens for the

study will be explained to the participants by one of us (A.H. Baqui) and informed consent will be obtained for the collection of specimens.

10. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

a) Background

Shigellosis is hyperendemic in Bangladesh and occasionally flares into epidemics (Hossain *et al*,1990). In routine hospitalized diarrhoeal cases, the isolation rate of *Shigella* is approximately 11-12% (Hossain *et al*., 1990). The disease maintains a regular seasonal pattern with two peaks, one in monsoon and the other in winter (Shahid *et al*., 1985; Khan and Mosley, 1968). It is not known how the seasonality and endemicity of this disease is maintained. The epidemiological behaviour of an organism depends mainly on how the organism adapts to the environment and the host. Thus, there are really two different ecosystems, one outside the human and the other inside the human host. So, we have to know how the organisms survive both inside and outside of human host. Therefore, detailed studies on survival of shigellae in human as well as on various surfaces of animate and inanimate objects need to be carried out. Because of limiting host parasite relationship, shigellae depend on direct or indirect person to person contact to perpetuate themselves in nature. Thus, maintenance of infection in the general population is related to personal hygiene and environmental sanitation. Epidemics occurring due to person-to-person transmission have been described (Nelson *et al*., 1967; Gangarosa *et al*., 1970).

A proper understanding of the existing ways of transmission of shigellae from environment to person and from person to person is lacking. To interrupt the possible means of transmission knowledge regarding the ability of animate and inanimate objects as vehicles is needed.

Hands

In person-to-person transmission, bacteria are mainly transmitted from contaminated hands to mouth. Inadequate hand-washing following anal washing after defaecation, washing of soiled clothes, etc. may cause shigellae to be transmitted via contaminated fingers to household objects, food and water, and ultimately to the mouth of susceptible individuals.

Several studies have demonstrated that hands may be important vectors for the transmission of bacterial pathogens (Salzman *et al.*, 1967; Lowbury *et al.*, 1970; Eisenach *et al.*, 1972; Rosendorf *et al.*, 1974; Parry *et al.*, 1980). Although *Vibrio cholerae* can survive and multiply up to 7 weeks in human sweat at 20-32°C (Dodin and Felix, 1972; Isaacson and Smith, 1979), it is not known whether shigellae can survive or multiply in hands.

Because organisms can be spread through the hands of personnel, hand washing following possible contamination has been recommended after toilet activities to prevent spread of enteric infections (Lowbury *et al.*, 1970; Eisenach *et al.*, 1972; Franco *et al.*, 1973; Sprunt *et al.*, 1970). Hand-washing with soap and water also helps to prevent nosocomial infection (Steere *et al.*, 1975). Black *et al.* (1981) carried out an epidemiological study in four day-care centres in the USA, to investigate the effect of hand-washing in the prevention of diarrhoea. They observed that the incidence of diarrhoea in hand-washing programme centres was reduced to half that of the control centers.

Likewise a prospective study in Dhaka, Bangladesh examined the effectiveness of a simple intervention, washing hands with soap and water (Khan *et al.*,

1952) and found that washing hands with soap and water could check the spread of shigellosis.

This study provided strong but indirect evidence that shigellae may be transmitted by contaminated hands as no attempts were made to culture organisms from hands.

Utensils

Shigellae are adapted to one host namely human. Primates and dogs are occasional victims of this organism (Mata, 1981). It is indeed difficult to explain the spread of shigellosis in large outbreaks due to person-to-person transmission alone, which suggests the existence of other mechanisms (Mata *et al.*, 1970; Pal, 1984). Transmission of shigellosis may depend on contamination of household surfaces. Children most commonly suffer from shigellosis and are, therefore, a major source of bacteria contaminating various household surfaces (Drasar, 1983), e.g. utensils, toys, etc. Moreover, mothers of infected children can also contaminate the domestic environment. However, to our knowledge, no study has been carried out yet to demonstrate the contamination of household surfaces with shigellae.

Clothes

Stanton and Clement (1986) conducted a study involving 247 families with 389 children under 6 years of age in 51 slum areas in Dhaka, Bangladesh. They investigated the extent of misuse of the sari with potential health hazards and correlated those uses with rates of childhood diarrhoea. They demonstrated an association between increased misuses of the sari and increased rates of diarrhoea. Misuses of the sari include cleaning utensils, hands, children, wiping eyes, blowing noses, and wiping washed and unwashed anuses of children. All these practices especially wiping unwashed anuses

makes the sari an important source of contamination. This study will attempt to isolate *Shigella* from saris of mothers which, by providing direct microbiological evidence for bacterial contamination, can help educate mothers.

Toilets and shower

Studies have demonstrated that the prevalence rate of *Shigella* excretion increases with increased uses of common latrine and shower facilities (Hollister *et al.*, 1955; Schliessmann *et al.*, 1958). Again, these studies only provide circumstantial evidence that *Shigella* may be acquired from common showers and toilet facilities as no attempt was made to isolate *Shigella* from the door-handles of bathrooms or latrines.

Milk bottles and nipples

Neonatal shigellosis has been reported by several authors (Haltalin, 1967; Salzman *et al.*, 1967; Whitfield and Humphries, 1967). Mata *et al.* (1969) studied a neonatal case of shigellosis in a Guatemalan village of Santa Maria Cauque. They suspected milk bottles as vehicles of transmission which were not handled hygienically or kept under proper sanitary conditions in the home.

Breast

Mata *et al.* (1969) also indicated that the source of shigellae in neonatal shigellosis may be mother's contaminated breast because these babies were strictly breast-fed in the first month of their lives. However, no attempt was made to isolate shigellae from mother's breasts.

Role of water in transmission of shigellosis

Survival of shigellae in various waters at various temperatures have been carried out (Feachem *et al.*, 1980; McFeters *et al.*, 1974; Talayara, 1960;

McGary and Stainforth, 1978). Surface water as a source of *Shigella* infection in Bangladesh has been documented by Rahaman *et al.* (1975). Khan *et al.* (1979) in a prospective study in Bangladesh observed that common water sources like ponds, lakes, canals may harbour different species of *Shigella* from where the disease can be contracted. Water stored at home for domestic use could be contaminated at the source of collection or at home with faeally contaminated hands or pitchers. Therefore, stored water need to be examined for shigellae.

Role of food in transmission of shigellosis

Food-borne outbreaks of shigellosis have been described in which potato salads, raw diced peppers, chicken salads, shredded lettuce, etc. have been implicated as vehicles of transmission (Weissman *et al.*, 1974; Donadio and Gangarosa, 1969; Davis *et al.*, 1988). In Bangladesh, foods such as rice, cooked fish, vegetables, lentil soup and fresh pineapples have been implicated as vehicles in one *Shigella* outbreak (Huq *et al.*, 1980). Therefore, leftover food needs to be investigated as vehicle of transmission of shigellosis.

Convalescent excretion

Convalescent excretion of shigellae by children up to a period of 8-10 weeks has been noted (Mata *et al.*, 1966). Earlier studies with adults have shown convalescent excretion after treatment with older drugs, such as streptomycin (Van Gelder *et al.*, 1947). This has been noted especially after treatment with inadequate dosages of the drug. There is, however, no information on the convalescent excretion of shigellae after treatment with newer drugs, such as nalidixic acid, ciprofloxacin and pivmecillinum. If a carrier state exists after treatment with these drugs, the individuals can very well transmit the pathogens to others in the household and the community.

Use of the polymerase chain reaction (PCR) technique

It is expected that shigellae will be in low concentrations on most surfaces contaminated by infected human faeces. Because, human faeces contain normal flora of the gut in greater numbers than shigellae, it becomes difficult to isolate shigellae due to overgrowth of normal flora in conventional culture media because no enrichment medium is available for shigellae. However, since the infective dose is very low (10-100 organisms, Levine, 1981), the low number of organisms which contaminate surfaces may be adequate to cause disease in a susceptible person. Therefore, a more sensitive technique is needed to detect the lower number of organisms from the environmental samples. Recently, the polymerase chain reaction (PCR) technique has enabled detection of shigellae even at low numbers (10 organisms in a sample) (Frankel *et al.*, 1990).

The PCR technique is capable of producing a selective enrichment of a specific DNA sequence by a factor of 10^6, greatly facilitating a variety of subsequent analytical manipulations (Saiki *et al.*, 1988). In this method a thermostable DNA polymerase is used in an *in vitro* DNA amplification procedure. PCR amplification involves two oligonucleotide primers that flank the DNA segment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences and extension of the annealed primers with DNA polymerase. These primers hybridize to opposite strands of the target sequence and are oriented so that DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of that DNA segment. Moreover, since the extension products are also complementary to, and capable of, binding primers, each successive cycle essentially doubles the amount of DNA synthesized in the previous cycle. This

results in the exponential accumulation of the specific target fragment, approximately 2^n, where n is the number of cycles.

Shigella produces diarrhoea by penetration and destruction of colonic epithelia (LaBrec *et al.*, 1964); this capacity is mediated in part by a large 120 to 140 Mdal plasmid, which carries the genetic determinants that encode epithelial cell invasion by each of the four species of *Shigella* and by closely related enteroinvasive *E. coli* strains (EIEC) (Sansonetti *et al.*, 1982). DNA probes have been developed corresponding to the large invasion plasmid (Small and Falkow, 1986; Buysse *et al.*, 1987) and used to identify *Shigella* and EIEC by nucleic acid hybridization. Recently, DNA sequences have been identified within an invasion-associated locus (ial) of the "invasion" plasmid that are specific for *Shigella* and EIEC (Frankel *et al.*, 1990). These sequences were shown to hybridize with the four *Shigella* species and with all tested EIEC.

Frankel *et al.* (1990) determined the DNA sequence of a 700-bp segment within the 2.5-kb *Hind*III fragment of the invasion plasmid (Small and Falkow, 1986); they have termed this segment the "invasion-associated locus" (*ial*). A unique region of *ial* was prepared as a 21 base synthetic oligonucleotide, conjugated to alkaline-phosphatase (AP) and tested for hybridization with EIEC and *Shigella* strains containing the virulence plasmid. Examination of the *ial* sequence led to the identification of two additional unique sequences flanking the sequence corresponding to the *ial* AP probe; these were used as primers for amplifying the 320 bp intervening sequence by PCR. They detected the amplified product by gel electrophoresis or DNA hybridization using the *ial* AP probe. They reconstituted normal stool specimens with various concentrations of *S. flexneri* and detected as few as 10 cfu by the *ial* AP probe after 26

cycles of amplification using this assay. They found that this assay was 10^2-fold more sensitive than the macrocolony hybridization assay using the same *ial* AP probe and 10^5-fold more sensitive than conventional biochemical tests for identifying enteric bacteria. Recently, this amplification assay was evaluated under field conditions in a rural region of Mexico using stool specimens from 71 children with diarrhoea. They found that PCR is substantially more sensitive than previously described tests, particularly when used during the course of epidemiologic investigations in developing countries.

PCR has also been used as a rapid method to detect enteroinvasive bacteria in food samples. Lampel *et al.* (1990) tested lettuce by artificially inoculating with 10^4 *S. flexneri*. Plasmid DNA was isolated from cultures of inoculated and uninoculated lettuce. With the PCR, the 760-bp fragment of invasive plasmid was generated only from lettuce inoculated with *S. flexneri*, as shown by gel electrophoresis and confirmed by southern blotting and by nucleotide sequencing of the amplified region. This assay performed by using PCR followed by gel electrophoresis, required less than one day and involved no radioactivity. There was no difference in sensitivity and specificity between radioactive probe and non-radioactive probe.

Previous studies had limitations of culturing shigellae from environment due to their low numbers and lack of suitable enrichment and culture media (Stypulkowska-Misiurewicz, 1981). By using PCR we will be able to overcome these difficulties.

Thus it is obvious that the role of various objects need to be vigorously studied for their definitive role in the maintainance and transmission of shigellosis using the most sensitive available technique (PCR) as well as the

conventional technique. In this study , we aim to get a direct proof of the involvement of these fomites in the transmission of shigellosis.

b) Research plan

Overall strategy

The study will be divided into two phases; a laboratory-based phase and a community-based phase.

i) Laboratory-based studies

This will involve studies directed at monitoring the survival of different species of *Shigella* on various utensils, clothes, hands, etc. at room temperatures. This study will be done by simple experiments involving the addition of a number of organisms to various animate and inanimate objects and the subsequent monitoring over a defined period of time. A suitable technique will be developed for these experiments as follows.

Preparation of inoculum: Trypticase soy broth (TSB) and MacConkey agar plates will be used for preparation of inoculum. 10^2-10^8 bacterial cells will be placed upon sterile cloth, wood (pieces of wood applicator sticks, 1 x 1 cm). The pieces of wood and cloth will be placed aseptically into petri dishes and will be stored at room temperature. Sampling of pieces of clothes or wood will be carried out at 0, 2, 4, 8, 12 and 24 hours duration for the 1st day and then at daily intervals. Using sterile forceps, the various pieces will be carefully removed and quantification done using standard plate-count procedures as well as the PCR technique. Experiments with each kind of samples will be repeated 3 times.

Examination of food: 10 g of solid food each will be taken in several petridishes. 10^2-10^8 cells of shigellae will be inoculated in each petri-dish. Sampling of food will be carried out at 0, 2, 4, 8 and 12 hours duration. In each sampling 10 g of food will be taken from a petri-dish and blended in a warring blender with 90 ml normal saline. Then appropriate 10-fold dilutions will be done in normal saline and quantification will be done using standard plate count procedure as well as PCR technique. Each food will be examined up to 12 hours. The laboratory-phased experiments will provide baseline data about the limit of detections of shigellae by the two techniques.

To determine whether PCR will detect the dead shigellae from the environmental samples, cloth, wood, food, etc. will be inoculated with shigellae and the shigellae in these objects will be killed by drying or by heating. Then PCR technique will be used to detect these dead shigellae.

Investigation of humans as carriers after antibiotic therapy: Only those patients with bacteriologically proven shigellosis attending the Clinical Research Centre of ICDDR,B will be studied. A sample of stool or R/S will be obtained daily for culture throughout the hospital stay of the patient. All specimens will be plated directly onto McConkey, XLD, SS and Hektoen agar media. Suspicious colonies will be examined by standard biochemical and serological techniques. The PCR technique will also be used to detect shigellae from stool and R/S samples. After discharge from the hospital, samples will be collected from the patient at home for convalescent excretion of *Shigella*.

Convalescent and asymptomatic carriers will be followed weekly until shedding of shigellae stops after testing five consecutive samples. An individual will be pronounced negative for excretion if *Shigella* organisms are not detected in 5 consecutive samples.

ii) Community-based studies

Selection of community: The field study will be carried out in Dhaka. The index family will be selected from the report of Dhaka hospital. A total of 240 cases will be followed over nine months.

Since family contacts (all members of the family including servants) of shigellae patients have a 20% chance of secondary infection with shigellae in the 10 days after a family member is hospitalized (Khan *et al.*, 1979), we propose to examine the role of various animate and inanimate objects in transmissoin of this disease among this high risk population.

Patients found to be positive by shigellae will be identified as index cases. Houses of the index cases will be visited within 24 h of admission and interviewed by trained workers about diarrhoea.

A family will be defined as a household i.e. all members live together and eat from the same cooking pot. A total of 1200 rectal swabs or stool samples will be taken from family members of index cases (240 index cases x 5 members), 240 samples from each category of animate and inanimate objects, e.g. flies, cockroaches, plates, glass, etc. will also be collected every alternate days for 10 days. All these samples will be examined by cultural and PCR techniques as described above. Any contact having clinically significant diarrhoea in the opinion of ICDDR,B staff will be encouraged to go to ICDDR,B

hospital. A person having both positive culture and symtoms (three or more loose motions in a day or, one or more loose stool with mucus/blood) will be defined as " case" and a person having only positive culture but no symptoms will be defined as an "asymptomatic carrier". As PCR will not distinguish shigellae detected from the index case and the environmental samples or contacts, analysis of plasmid profiles of the strains of index cases and the strains detected by PCR from the environment and contacts will be carried out.

Sample size:

For calculation of sample size for convalescent carrier and environmental samplings, it is assumed that 10% of the patients will develop convalescent carrier state.

If 95% confidence limit of the estimated carrier state lies between 0.06-0.14, we will consider the true carrier state is around 0.1.

To detect this, we need n patient where $n = \frac{(Z_\alpha)^2 (pq)}{d^2}$

$p=0.1$, $q=(1-p)=0.9$, $d=0.04$, $n = \frac{(1.96)^2 \times 0.1 \times 0.9}{(0.04)^2} = 216$

It is expected that there will be about 10% dropouts. Therefore, (216+21) = 237 patients need to be studied. However we will study 240 patients for convalescent carrier state as well as for environmental samplings.

The animate and inanimate objects and the household contacts of these ~~70~~ 240 index cases will be studied. Data will be analysed to examine the possible correlation between household fomites contamination and secondary attack rates.

Hands (n=1200): Hands of adult patients with shigellosis in the hospital will be washed with normal saline and after visiting the toilet (Pinfold *et al.*, 1988) and the washing will be examined for *Shigella* by culture and PCR. In case of children, mothers and other relatives who wash the anus of the child will be examined following both PCR and cultural technique. Stool sample or rectal swab from the secondary cases from close contacts of index patients will be transported to the laboratory in Cary & Blair transport media. Hand washings will be transported to the laboratory in buffered glycerol saline in a foam-box with ice packs.

Inanimate objects: Inanimate objects, e.g. plates, glass, spoon, milk bottles and its nipples, toys, water pot, sari, etc. will also be washed with normal saline and examined for shigellae following the above procedures. Samples will also be cultured for faecal coliforms as an indirect way of assessing faecal contamination.

Door-handle (n=240): Door-handles will be swabbed by using sterile cotton swabs moistened with sterile saline. Shigellae will be looked for by culture and PCR.

Breast (n=100): Breasts of convalescent mothers and the mothers of breast-feeding children with shigellosis will be washed in normal saline and washings will be tested for *Shigella* by culture and PCR.

Water (n=240): Samples will be collected from water at home and surface water which is used for drinking and other purposes. Water will be collected in pre-sterilized 500 ml plastic bottles. All the collected samples will be transported to the laboratory inside an insulated foam box with ice bags.

500 ml aliquots of water will be filtered through 0.45 µm membrane filters. The filter paper will be placed onto MacConkey, XLD, SS and Hektoen agar and

incubated for 24 hours. Faecal coliform will be counted to investigate the level of faecal contamination. For coliform count, MFC agar will be used and rest of the procedures will be followed as described in standard methods (APHA, 1985).

Food: Leftover food will be collected from the houses of the index families and transported to the laboratory. Then the foods will be examined following both cultural technique and PCR as described above.

All the environmental samples will be collected every alternate days for 10 days and will be processed within 6 hr of collection.

Procedure for detection of shigellae by PCR

Samples: Stool, R/S and environmental samples will be analysed by PCR technique.

Stool: 200 mg of the stool sample will be suspended in 1.0 ml of PBS, pH 7.4. Insoluble particulate matter will be removed by low speed centrifugation and the bacteria in the supernatant will be obtained by centrifugation for 1 min in an Eppendorf microcentrifuge. Then the pellet will be used for plasmid DNA extraction.

R/S sample: R/S sample will be suspended in 1.0 ml PBS, pH 7.4. Then the suspension will be centrifuged and the pellet will be used for plasmid DNA extraction.

Environmental samples: The swab washing and washing from the animate and inanimate objects will be centrifuged and the pellet will be used for plasmid DNA extraction.

Extraction of plasmid DNA: The pellet will be resuspended in 75 µl of 50 mM Tris-HCl buffer, pH 8.0, containing 20% (w/v) sucrose, 50 mM EDTA and 100 µg of lysozyme and the reactants will be incubated at 37°C for 30 min. Then 300 µl of 50 mM NaCl containing 1% (w/v) sodium dodecyl sulphate (SDS) and 800 µg of proteinase K will be added and the incubation will be continued for an additional 60 min. The extracted DNA will be precipitated by adding of absolute ethanol harvested by centrifugation and then resuspended in 100-300 µl of 10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA. Then the extracted DNA will be stored in distilled H_2O at 4°C until needed.

Analysis of plasmid DNA: The extracted plasmid DNA will be separated by vertical gel electrophoresis in 0.7% agarose (Meyers *et al.*, 1976). The gels will be stained in ethidium bromide solution (0.5 µg/ml) for 30 mins and will be destained overnight at 4°C (Haider *et al.*, 1988). Photographs will be taken with an MP4 Land camera with type 55 P/N film and a No. 22 Wratten gelatin filter (Eastman Kodak Co,, Rochester, N.Y.) with UV transillumination. The molecular size of the plasmid DNAs of the isolated strains from the environment and contacts will be determined on the basis of its mobility through agarose gel and will be compared with the mobility of known molecular size plasmids of the strains isolated from index cases.

Amplification of DNA by PCR: 1-5 µl of the extracted DNA will be amplified by PCR using 0.2 µg of each of the primers following procedures described by Saiki *et al.* (1986, 1988). The sequence of the primers are 3'-GGA GTG GTA TGG ATG GTC 5' and 5'-CCA GGC CAA CAA TTA TTT CC-3' (Frankel *et al.*, 1990). PCR will be performed for 26 cycles of 1 min at 94°C (denaturation), 2 min at 43°C (annealing of primers to single stranded DNA) and 3 min at 72°C (DNA polymerase mediated primer extension). Then 10 µl of the amplified product

will be analyzed by electrophoresis using 1.5% agarose gels. The identity of the amplified product will be further confirmed by a probe as described below.

Detection of PCR-amplified products with the alkaline phosphatase labeled oligonucleotide *ial* probe (*ial* AP probe)

Ten µl of PCR-amplified reactants (as described above) will be analyzed by slot blot hybridization with the *Shigella ial* probe. The sequence of the *ial* probe is 3'-GTG TCC ATA AGA TTA TCT ACC 5'- (Frankel *et al.*, 1990). Before hybridization, the DNA will be incubated in 0.3 M NaOH for 5 min at 70°C and will be applied to a nylon membrane. Before hybridization the membrane will be incubated in 5X SSPE, containing 5X Denhart's, 0.5% SDS and 0.1 mg/ml yeast tRNA for 15 min at 42°C. Then 4 ng of the AP probe will be added and hybridization will be allowed to proceed for 15 min. Then the membrane will be washed four times at room temperature. 2X SSC, 0.5% SDS for 10 min; 1X SSC, 0.1% SDS for 10 min; 1X SSC, 0.5% Triton X-100 for 5 min, 1X SSC for 1 min. Then the membranes will be incubated with a substrate for the AP probe consisting of a mixture of nitro blue tetrazolium (NTB) and 3-bromo-4-chloro-3-indolyl phosphate (BCIP) for 4 hr at room temperature.

The primers and alkaline phosphatase labelled probe will be made and supplied by Dr. Denis Kopecko, Walter Reed Army Institute of Research, Washington D.C., U.S.A.

Interpretation of data

Isolation of shigellae in culture and/or the detection of target sequences in the sample will be considered evidence of contamination of the surfaces by shigellae. If the higher rate of detection of shigellae from the environment by PCR correlate with the higher secondary attack rate among the family members of index case it will give an idea about the role of environment in

transmiting the disease. The index case may contaminate the environment from where the family members may acquire the disease. If PCR does not detect shigellae from the environment, possibilities are that there is no environmental transmission of shigellae or the environment has not been adequately sampled.

c) Bibliography

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11. PUBLICATIONS OF PRINCIPAL INVESTIGATORS

Dr. Md. Sirajul Islam

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16) Islam MS and Drasar BS. A review on aquatic flora as possible reservoirs of toxigenic *Vibrio cholerae* 01 in the aquatic environment. Tropical Diseases Bulletin. (Submitted).

17) Islam MS and Alam MJ. Freshwater pond ecosystems as a reservoir of *Vibrio cholerae* 01 in an endemic area of Bangladesh. Transactions of the Royal Society of Tropical Medicine and Hygiene. (Submitted).

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Dr. M. J. Albert

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14) Albert MJ. Epidemiology of rotavirus infection in children in Indonesia. In: Kurstak E and Thongcharoen P (eds.) Viruses and diseases in Asia. Mahidol University, Bangkok, Thailand 1988:262-255.

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17) Albert MJ, Singh KV, Murray BE and Erlich J. (1990). Molecular epidemiology of shigella infection in Central Australia. Epidemiology and Infection 105:51-58.

18) Hossain MA, Albert MJ and Hasan A. (1990). Epidemiology of shigellosis in Teknaf, a coastal area of Bangladesh: a 10 year survey. Epidemiology and Infection 105:41-50.

19) Albert MJ, Kibriya AKMG and Tzipori S. (1990). Reliability of colony characteristics on MacConkey agar in the identification of *Escherichia coli* for epidemiological investigations. Journal of Diarrhoeal Disease Research 8:166-167.

20) Albert MJ, Salam A, Qadri F, Kibriya AKMG and Tzipori S. (1991). Simultaneous infection with multiple serotypes of shigellae in a patient. Diagnostic Microbiology and Infectious Disease (in press).

21) Albert MJ, Hossain MA, Alam K, Kabir I, Neogi PKB and Tzipori S. (1991). A fatal case associated with *Vibrio fluvialis* septicaemia. Diagnostic Microbiology and Infectious Disease (in press).

22) Hossain MA and Albert MJ. (1991). Effect of duration of diarrhoea and predictive values of stool leukocytes and red blood cells on the isolation of different serogroups/ serotypes of shigellae. Transactions of the Royal Society of Tropical Medicine and Hygiene (in press).

23) Albert MJ, Ansaruzzaman M, Neogi PKB, Haider K, Faruque SM and Tzipori S. An enzyme-linked immunosorbent assay for the detection of localised adherent enteropathogenic *Escherichia coli* serogroups. Submitted for publication.

24) Albert MJ, Alam K, Islam M, Montanaro J, Rahman H, Haider K, Hossain MA, Kibriya AKMG and Tzipori S. *Hafnia alvei*: a probable diarrhoeal pathogen of humans. (submitted for publication.

25) Faruque SM, Haider K, Albert MJ, Ahmed QS, Nahar S and Tzipori S. A comparative study of gene probes and standard bioassays to identify diarrhoeagenic *Escherichia coli* in Bangladeshi paediatric diarrhoeal patients. Submitted for publication.

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28) Albert MJ, Leach A, Asche V, Hennessy J and Penner J. Epidemiology of *Campylobacter jejuni* and *Campylobacter coli* infections in hospitalised patients in Central Australia. Submitted for publication.

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31) Albert MJ, Alim ARMA, Ansaruzzaman M and Mitra A. Fluorescent antibody test for rapid diagnosis of *Shigella dysenteriae* 1 infection. Submitted for publication.

12. FLOW CHART

Activities of the Laboratory, 1-2 years

First year : Setting up methodology and field-sampling.

: Experiments with animate and inanimate objects in the laboratory settings.

Second year : Field-sampling and writing.

13. ITEMIZED SPECIFIC TASKS FOR EACH LISTED INVESTIGATORS

Sequence of tasks

Standardization of procedures] 3 months

↓

Testing of specimens] 18 months

↓

Analysis of data and writing reports] 3 months

Task of investigators

Investigator	Task
M. S. Islam M. J. Albert	Standardization of procedures
M. J. Albert M.S. Islam	Setting up PCR technique and screening of samples
M. S. Islam A. H. Baqui	Field investigation
M.S. Islam M.J. Albert A. Felsenstein	Data analysis
Medical Officer	Identification of patients with shigellosis

MSI:mh/S10:SHIGLOS2.PRO

14. BUDGET

BUDGET SUMMARY

First year

Capital equipment for PCR	US$	10,000
Personnel		8,328
Operating cost		14.800
	US$	33,128

Second year

Personnel	US$	12,141
Operating cost		73,000
	US$	85,141

Total cost (for 2 years) : US$ 33,128 + 85,141 = US$ 118,269

DETAILED BUDGET

First year

A. Personnel

Designation	Level & step	Salary per annum
Research Officer	GSV-1	US$ 5,400
Laboratory Technician	GSIII-1	2,928
		8,328

B. Operating costs

Experiments in the laboratory:

a)	Expts. with wood, clothes metal, glass, water	...	5,000
b)	Handwashing	...	3,000
c)	Expt. with food	...	1,000
d)	Primers and probe	...	800
e)	Stationary and laboratory supplies	...	5,000
		US$	14,800

Total cost (for first year) : US$ 8,328 + 14,800 = US$ 23,128

Second year

A. Personnel

Designation	Level & step	Salary per annum
Research Officer	GSV-1	US$ 5,528
Laboratory Technician	GSIII-1	3,013
Field worker	GSIII	3,600
		US$ 12,141

B. Operating costs

Environmental sampling:

a)	Sampling of cloth and other household surfaces	...	US$ 12,000
b)	Handwashing	...	12,000
c)	Breast washing	...	1,000
d)	Monitoring *Shigella* excretion by patients	...	12,000
e)	Sampling of food	...	12,000
f)	Field transport	...	8,000
g)	R/S samples of carrier	...	12,000
h)	Faecal coliform estimation	...	1,000
i)	Stationary and laboratory supplies	...	3,000
			US$ 73,000

Total cost (for second year) : US$ 12,141 + 73,000 = US$ 85,141

MSI:mh/S10:SHIGLOS2.PRO

CONSENT FORM

Yesterday a member of your family became sick with dysentery and was hospitalized at the Cholera Hospital in Dhaka. In the next few days, other members of your family may become sick with dysentery as well. The doctors of cholera hospital are studying how this disease can be prevented. The germs of this disease may persist in various utensils and household surfaces from where they can be ingested and cause dysentery. So we will swab various utensils and household surfaces with a cotton swab moistened with sterile normal saline. The breast fed babies can ingest these germs from mother's breast if mother's breask is faecally contaminated. So mother's breasts will be washed with water and this water will be examined in the laboratory to see whether or not this washed water contains the germs of dysentery. We will give you medicine to be cured from dysentery. We will visit your family every alternate days for 10 days, inquire about any illness and take a rectal swab which will help us to determine if others have dysentery.

If you or anyone should become sick during this period, we will either provide treatment for you at home or take you to the hospital.

During this study and afterwards, your names and any information on illness that you provide will be held confidential. You will not be specifically named or identified in connection with this study. If you choose not to participate, you may still receive treatment at the Cholera Hospital should you become ill. If you do not agree to participate, you may withdraw from the study at anytime.

If you are willing to participate in this study, please sign or put your left thumb print below.

_____ _____

Signature or left thumb print Date

_____ _____

Signature of investigator Date

_____ _____

Signature of witness Date

REVIEWER NO. 1

Project title: Investigation of the carrier state and the role of animate and inanimate objects as reservoirs or secondary hosts of shigellae

Principal Investigator(s): .....

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project		✓	
Adequacy of Project Design			✓
Suitability of Methodology		✓	
Feasibility within time period		✓	
Appropriateness of Budget			
Potential value to field of knowledge			✓

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification:
- on technical grounds ☒
- on level of financial support ☐

I do not support the application ☐

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel they are justified.

(Use additional pages if necessary)

The study proposes to use PCR to study convalescent carriage and environmental contamination with Shigella. Although likely more sensitive than culture, there may be problems to be overcome before the full potential of this technique can be realized.

First, it is possible that PCR will detect the genetic sequences of dead shigellae. If so the carriage studies may not be compromised, but the environmental studies would be. Detection of such noninfectious material would confuse the analysis. Laboratory studies should include PCR studies of objects, food, etc., where the organisms have died because of drying on surfaces, heating, etc. If PCR cannot discriminate living from dead shigellae, it will not be of much use for transmission studies.

Second, this method cannot distinguish one Shigella organism from another. It is certain that there will be many Shigellae in the environment that did not come from the index case. This was a problem with family studies of Shigella in Peru, where family members had a high rate of infection, but with types of Shigella different from the index care. If this cannot be done by PCR, it will limit its use. Molecular epidemiologic techniques, eg., restriction digests, plasmid profiles, could be done on isolates but are not proposed.

If the first two concerns can be overcome there are a number of lesser concerns with the proposal. Although of use for descriptive purposes, the testing of hypotheses about transmission if very weak. The relationships between infection of cases and family members and of objects is not well-conceptualized and no analysis is proposed, nor is a sample size calculation for examining the risk factors for secondary infections. The sampling of the environment is also not well described (which objects will be tested in a given hour, how often, how many foods and when to culture, etc).

There is no reaction on analysis and that on interpretation (one sentence). Suggesting that the outcomes of the study have not been carefully conceptualized.

REVIEWER NO. 2

Project title: Investigation of the carrier state and the role of animate and inanimate objects as reservoirs or secondary hosts of shigellae

Principal Investigator(s): .....

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project		x	
Adequacy of Project Design			x
Suitability of Methodology		uncertain	
Feasibility within time period		uncertain	
Appropriateness of Budget	x		
Potential value to field of knowledge	x		

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification:
- on technical grounds ☐
- on level of financial support ☐

I do not support the application ☒

DETAILED COMMENTS

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel they are justified.

(Use additional pages if necessary)

REVIEWERS COMMENTS

Reviewer No. 1

1) As suggested by reviewer, experiments with dead shigellae will be carried out by PCR which has been incorporated in the protocol, page 12, para 2.

   Even if PCR cannot distinguish between live and dead shigellae, it will detect the genetic sequences which will indicate that shigellae were present in the surface of a particular object which may act as potential source of transmission.

2) As suggested by the reviewer, plasmid profiles of shigellae detected by PCR will be carried out which has been incorporated in the protocol, page 14, para 1.

   The sample size for examining secondary infection has been calculated and incorporated in the protocol, page 13, para 5.

   The sample size for inanimate objects has also been calculated and incorporated in the protocol, page 13, para 5.

   Sampling of the environment has been described, page 16, para 3.

   Interpretation of results has been modified, page 18, last para.

Reviewer No. 2

After incorporating the comments made by Reviewer No. 1, the project design is modified.

Regarding suitability of methodology, suggestions given by Reviewer No. 1 has been incorporated, page 12, para 2, and page 14, para 1.

Feasibility within time period ranked as uncertain. However, we will be able to extend the time period of the protocol if needed.

Reviewer I
Dr. Brooyne Islam protocol

Project title: Investigation of the carrier state and the role of animate and inanimate objects as reservoirs or secondary hosts of shigellae

Principal Investigator(s): .....

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project		✓	
Adequacy of Project Design			✓
Suitability of Methodology		✓	
Feasibility within time period		✓	
Appropriateness of Budget			
Potential value to field of knowledge			✓

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification:
- on technical grounds ☒
- on level of financial support ☐

I do not support the application ☐

Name of Referee: Robert Black

Position: Professor, International Health

Institution: J. HU School of Public Health

Robert Black
Signature

2-24-91
Date

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel they are justified.

(Use additional pages if necessary)

The study proposes to use PCR to study convalescent carriage and environmental contamination with Shigella. Although likely more sensitive than culture, there may be problems to be overcome before the full potential of this technique can be realized.

First, it is possible that PCR will detect the genetic sequences of dead shigellae. If so the carriage studies may not be compromised, but the environmental studies would be. Detection of such noninfectious material would confuse the analysis. Laboratory studies should include PCR studies of objects, food, etc., where the organisms have died because of drying on surfaces, heating, etc. If PCR cannot discriminate living from dead shigellae, it will not be of much use for transmission studies.

Second, this method cannot distinguish one Shigella organism from another. It is certain that there will be many Shigellae in the environment that did not come from the index case. This was a problem with family studies of Shigella in Peru, where family members had a high rate of infection, but with types of Shigella different from the index care. If this cannot be done by PCR, it will limit its use. Molecular epidemiologic techniques, eg., restriction digests, plasmid profiles, could be done on isolates but are not proposed.

If the first two concerns can be overcome there are a number of lesser concerns with the proposal. Although of use for descriptive purposes, the testing of hypotheses about transmission if very weak. The relationships between infection of cases and family members and of objects is not well-conceptualized and no analysis is proposed, nor is a sample size calculation for examining the risk factors for secondary infections. The sampling of the environment is also not well described (which objects will be tested in a given hour, how often, how many foods and when to culture, etc).

There is no reaction on analysis and that on interpretation (one sentence). Suggesting that the outcomes of the study have not been carefully conceptualized.

Reviewer 2
Dr. Siraj ul Islam's proposal

Project title: Investigation of the carrier state and the role of animate and inanimate objects as reservoirs or secondary hosts of shigellae

Principal Investigator(s): ....

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project		x	
Adequacy of Project Design			x
Suitability of Methodology		uncertain	
Feasibility within time period		uncertain	
Appropriateness of Budget	x		
Potential value to field of knowledge	x		

CONCLUSIONS

I support the application:

a) without qualification /__/

b) with qualification:
- on technical grounds /__/
- on level of financial support /__/

I do not support the application /x/

Name of Referee: Herbert L. DuPont, M.D.

Position: Professor and Director, Center for Infectious Diseases

Institution: The University of Texas Medical School/School of Public Health

[signature]
Signature

3/26/91
Date

DETAILED COMMENTS

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel they are justified.

(Use additional pages if necessary)

I think this could be an outstanding study. Using PCR to study the family and environmental transmission of Shigella is a good idea. The problems with this proposal are:

1) No preliminary data are presented to indicate the PI can perform PCR.
2) No details of the methods are provided: how many food, water, breast, and inanimate objects will be cultured, how will they be selected, etc.?
3) Inadequate documentation of the PI's capabilities were provided.