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Survival of *Shigella dysenteriae* Type 1 on Fomites

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ABSTRACT

Studies have shown that various objects, such as utensils, toys, and clothes, can serve as vehicles for transmission of *Shigella* spp. Shigellae can become viable but non-culturable (VBNC) when exposed to various environmental conditions as shown in earlier studies. The present study was carried out to detect VBNC *Shigella dysenteriae* type 1 on various fomites by direct viable counting, polymerase chain reaction (PCR), and fluorescent antibody methods. *S. dysenteriae* type 1 was inoculated onto cloth, wood, plastic, aluminum, and glass objects. Results showed that 1.5-4.0 hours after inoculation, *S. dysenteriae* type 1 became non-culturable, and after five days, non-culturable but viable *S. dysenteriae* type 1 could be detected by both PCR and fluorescent antibody techniques. Fomites can be considered an important potential route of transmission of VBNC *S. dysenteriae* type 1 and a significant factor in the epidemiology of shigellosis.

Key words: *Shigella dysenteriae*; Dysentery, Bacillary; Disease transmission; Diagnosis, Laboratory; Bangladesh

INTRODUCTION

Shigellosis occurs worldwide, but its incidence and prevalence are greater in developing countries with high morbidity and mortality. In Bangladesh, the disease is hyperendemic and occasionally flares into epidemics. The isolation rate of *Shigella* in routine surveillance of hospitalized diarrhoeal cases is approximately 11-12% (1).

Transmission of shigellosis, in general, occurs via personal contact or fomites. In the case of the latter, utensils used in eating and drinking glasses are important vectors in the transmission of the disease. A study of the transmission of *Shigella sonnei* among school children found that the organism could be isolated from toilet seats, toilet floors, clothes, bedding, toys, and floors of homes (2). Among enteric pathogens, shigellae are transmissible to humans and infective in very small numbers varying from a few to a few thousand, depending on the species (3). Contamination of children's hands has been correlated with contamination of toy-balls and other inanimate objects in a day-care centre (2).

The capability of pathogenic micro-organisms to exist in the viable but non-culturable (VBNC) state has been reported (4-6). Therefore, the potential health hazard of *Shigella* species existing in the VBNC state may be important, since *Vibrio cholerae* O1 could be isolated in the culturable form from stools of volunteers after ingestion of VBNC *V. cholerae* O1 (7). Furthermore, some investigators claim that non-

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culturable bacteria of selected species can be resuscitated to the culturable state (8).

A significant problem in elucidating the potential hazard of non-culturable pathogenic bacteria is the inability to detect such cells in the natural environment by routine bacteriological culture methods. The fluorescent antibody (FA) technique, a highly-selective and sensitive method, can detect VBNC shigellae in laboratory microcosms (5). The FA technique can also detect and identify bacteria in the natural environment (5,9-11) and in microcosms (12). The technique can as well detect VBNC bacteria in the environment, even when such bacteria are present in low numbers (13,14).

Using polymerase chain reaction (PCR), a specific segment of DNA can be amplified by a factor of 10^6 or more within hours (15). The method requires only the presence of the target DNA to detect non-culturable cells. Thus, a combination of direct viable count (DVC) and PCR is a suitable method to detect VBNC bacteria.

Although inanimate objects play an important role in the transmission of shigellae, little information on the survival of *Shigella* on contaminated inanimate objects is available. Drasar found that children are the most frequent victims of shigellosis and are a significant source of bacteria contaminating various household surfaces, including utensils, toys, etc. (16). For example, using conventional culture techniques, he observed that *S. sonnei* could survive up to 17 days on a wooden chair in a temperate climate. Nakamura reported that *S. sonnei* could survive 10–57 days on the surfaces of metal, wood, paper, glass, cotton, etc. at –20 °C (2). However, no studies, using FA and PCR techniques, were conducted to generate data on the survival of *S. dysenteriae* type 1 on such objects in tropical climates. Using conventional culture, FA and PCR techniques, this study was carried out to determine how long *S. dysenteriae* type 1 could survive on various inanimate objects (fomites).

MATERIALS AND METHODS

Survival experiments

The experiments carried out in this study were carefully designed, including inoculation of fomites (1x1 cm^2) with test organisms and subsequent monitoring of viable cells over a defined period of time. *S. dysenteriae* type 1 strain AE 21725, obtained from the Clinical Microbiology Laboratory of ICDDR,B: Centre for Health and Population Research, was used in this study. The culture was first inoculated onto MacConkey agar and incubated at 37 °C for 24 hours. A loopful of growth was suspended in 10 mL phosphate-buffered saline (PBS) adjusted to 90% transmittance of the suspension at 585 nm wavelength of a spectrophotometre (Colman Junior, IIA model 6/20A, Perkin Elmer Corporation, USA). Bacterial counts were obtained as described by Islam *et al*. (14).

Inoculation of fomites

Inanimate objects, employed in this study, included cloth, wood, plastic, aluminum, and glass. Samples of the objects were cut into small pieces (1x1 cm^2), and the pieces were autoclaved. Sterile pieces of fomites were aseptically arranged in sterile covered petri dishes and allowed to dry.

Inoculation and incubation of fomites

Fifteen to 20 pieces of each kind of fomite were placed in each petri dish, after which 10 µL of PBS containing approximately 10^5 cfu of *S. dysenteriae* type 1 were inoculated onto each piece. All inoculated materials in the covered petri dishes were incubated at a temperature of 25 °C in the dark.

Sampling times and counting procedure

Culturability of *S. dysenteriae* type 1 was tested at pre-determined time intervals by plate count. Culturable cells were enumerated at 30-minute intervals up to 6 hours and again after 24 hours. At each sampling, four pieces of each fomite were aseptically transferred into four separate test-tubes, each containing 1 mL of PBS. The test-tubes were vortexed vigorously for one minute, and 10-fold dilutions were prepared in PBS, after which 25 µL of bacterial suspension from different dilutions were plated on MacConkey agar, using the drop plate method. The plates were incubated at 37 °C for 18-24 hours and the bacterial colonies counted, with results expressed as colony-forming units per cm^2 (cfu/cm^2). When no culturable cells were obtained after two consecutive samplings, pieces of fomite were placed in Gram-negative broth, incubated overnight, and plated on MacConkey agar, incubated at 37 °C for 18-24 hours.

DNA preparation

A piece of inoculated fomite from which no culturable *Shigella* was obtained was placed in 10 mL of PBS in a test-tube and vortexed for one minute. Then 1 mL of the suspension was transferred to a microfuge tube and centrifuged at 15,000 x g for 10 minutes. The supernatant was collected, and DNA of the bacterial cells was extracted by the method described by Islam *et al*. (5)

DNA amplification by PCR

The amplification of *ipa*H sequences was performed in 100 µL of reagent in polypropylene microfuge tubes

(Perkin-Elmer Cetus, Norwalk, Conn., USA) (17,18). The reagent was prepared using 10 µL of buffer solution, 2 µL each of deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 0.5 µL of Taq DNA polymerase, 1 µL of extracted DNA with 120 ng each of two primers (primer H8 (5'-GTTCCTTGACCGCCTTTCCGATAC-3'] and H15 [5'-GCCGGTCAGCCACCCTC-3'] derived from an invasion plasmid of *S. flexneri* M90T, 8 µL of extracted DNA, and sterile deionized water to a final volume of 100 µL.

The reagent was overlaid with 50 µL of mineral oil. PCR was carried out for 35 cycles of one minute at 94 °C (for denaturation), 1.5 minutes at 60 °C (for annealing of primers to single-stranded DNA), and 0.25 to 1 minute at 72 °C (for DNA polymerase-mediated extension). Amplified DNA was separated electrophoretically on 0.8% agarose gel and transferred to a nylon membrane (Sigma) by capillary action.

DNA hybridization

DNA-containing nylon membranes were prehybridized in pre-warmed hybridization buffer (5xSSC [1xSSC=0.15 M NaCl and 0.015 M sodium citrate], 1.0% SDS, 0.5% bovine serum albumin) for 10 minutes at 37 °C. Hybridization was carried out using the same pre-hybridization buffer. The membrane was incubated at 37 °C for 30 minutes in a water-bath. The probe was the same fragment as that amplified using primer H8 and H15. Hybridization was carried out, according to the ECL (enhanced chemiluminescence) direct nucleic acid labelling and detection systems protocol (Amersham LIFE SCIENCE, Amersham International plc, Amersham Place, Little Chalfont, Buckinghamshire, England HP7 9NA), following the procedure described by Jablonski *et al.* (19).

The membranes were transferred to a solution of 7.5 mL alkaline phosphatase buffer composed of 100 mM Tris HCl, 100 mM NaCl, 50 mM $MgCl_2$, 0.1 mM ZnCl, and 0.02% sodium azide (pH 8.5). The buffer contained 33 µL of nitroblue tetrazolium (Sigma) solution (75 mg/mL in 70% dimethyl formamide) and 25 µL of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) solution (50 mg/mL in dimethyl formamide). The reagent was incubated at room temperature overnight in the dark and terminated by washing the filters with distilled water.

Fluorescent microscopy and viability testing

A piece of inoculated fomite from which no culturable *S. dysenteriae* was obtained was placed in 10 mL PBS in a test-tube and vortexed for one minute. Then 1 mL of the suspension was transferred to a microfuge tube and centrifuged at 3,000 x g for 10 minutes. The supernatant was collected and centrifuged at 15,000 x g for five minutes. The supernatant was discarded, and the pellet was resuspended in 25 µL PBS (pH 7.3). Five µL of the suspension was applied to a microscopic slide. The bacteria were stained with *S. dysenteriae* type 1 polyclonal antiserum (Wellcome Diagnostics, Dartford, UK) and fluorescein isothiocyanate-conjugated (FITC) anti-rabbit goat serum. A cover slip was placed on the slide with buffered glycerol (pH 8.3) and then examined under an epifluorescence microscope (Olympus Model BH-2). *S. dysenteriae* type 1 was used as a positive control. Viability testing of non-culturable *S. dysenteriae* type 1 was carried out according to the procedure described by Kogure *et al*. (20). In brief, non-culturable cells are added to a solution containing yeast extract (0.025%) as a nutrient supplement and nalidixic acid (0.002% as an antibiotic which inhibits cross-wall formation upon cell division by inhibiting DNA gyrase and terminating DNA polymerization. Hence, viable cells will start growing, but will not be able to divide. In this procedure, viable cells appear elongated compared to their normal length.

RESULTS

Culturability of *S. dysenteriae* type 1 on different fomites is shown in Fig. 1, with different survival times obtained for the fomites. Among the five materials, the longest and shortest survival times during which culturable cells could be detected were: plastic < glass < aluminum < wood < cloth (Fig. 1). *S. dysenteriae* type 1 remained culturable longest on cloth. All materials were dry during exposure times, so that residual moisture did not contribute to culturability.

After inoculation, DNA was prepared from non-culturable *S. dysenteriae* type 1 exposed on the dry fomite surfaces after five days, and PCR was applied, after which the products were analyzed by agarose gel electrophoresis. DNA was transferred to nylon membranes and hybridized against the *ipa*H probe. All fomite surfaces containing non-culturable *S. dysenteriae* type 1, generated the expected 700-bp fragment of the H locus, which was hybridized to the *ipa*H probe (Fig. 2 and 3).

At the same time as PCR was done, the fluorescent antibody direct viable count (FA DVC) method was used for detecting VBNC *S. dysenteriae* type 1. VBNC *S. dysenteriae* type 1 was present on all the fomites, i.e.

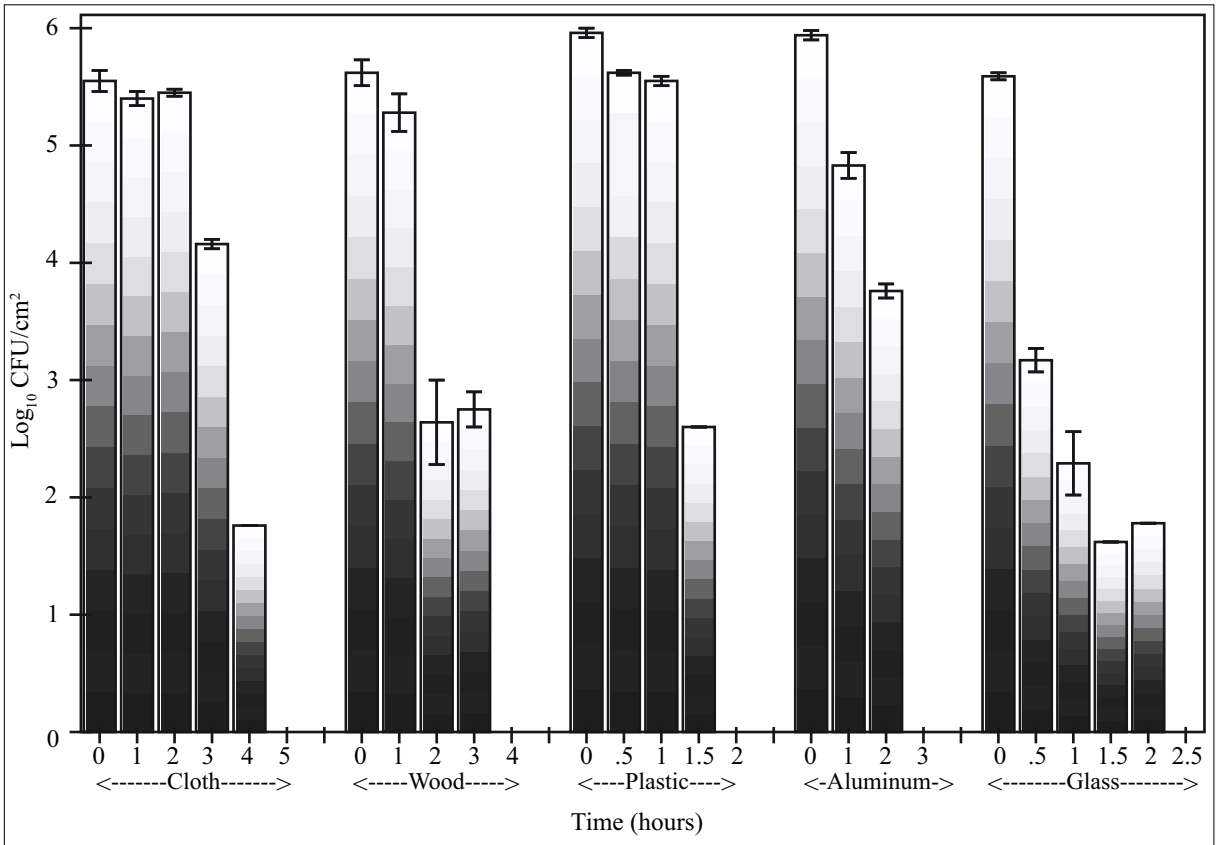

Fig. 1. Culturability of *S. dysenteriae* type 1 (21725) from fomites on MacConkey agar

enlarged cells were present after treatment. It was observed that *S. dysenteriae* type 1 loses culturability on fomite surfaces employed in this study between 1.5 and 4 hours after direct inoculation, since culturable cells could be recovered only up to 1.5 hours from plastic after inoculation and up to 4 hours from cloth.

DISCUSSION

Where hygiene is poorly-maintained, contamination of food and/or drinking water constitutes the most significant mode of transmission. Also, transfer via human faeces can occur between mother and child due to lack of good hygienic practice. In a developing country, like Bangladesh, transmission through fomites can also be an important mode of infection. The ability of *S. dysenteriae* type 1 to enter the VBNC state on fomites complicates the situation. As a mechanism of survival, as suggested by Roszak and Colwell (21), the VBNC strategy perpetuates the organism after exposure to conditions less than optimal for cell growth and division.

In conclusion, *S. dysenteriae* type 1 was viable for significant periods of time on different fomites, with VBNC cells detectable on cloth, wood, plastic, aluminum, and glass surfaces five days after inoculation. The FA and PCR results were consistent with those of other studies where *V. cholerae*, *Campylobacter* spp., *Escherichia coli*, and *S. flexneri* failed to grow in conventional culture media, but remaining viable, after exposure to conditions not conducive to active growth and cell division (4,9,22). In developing countries, like Bangladesh, where poor hygienic conditions prevail and contact with contaminated household surfaces occurs frequently, *S. dysenteriae* type 1 in the VBNC state on fomites may be a potentiator in the transmission of shigellosis.

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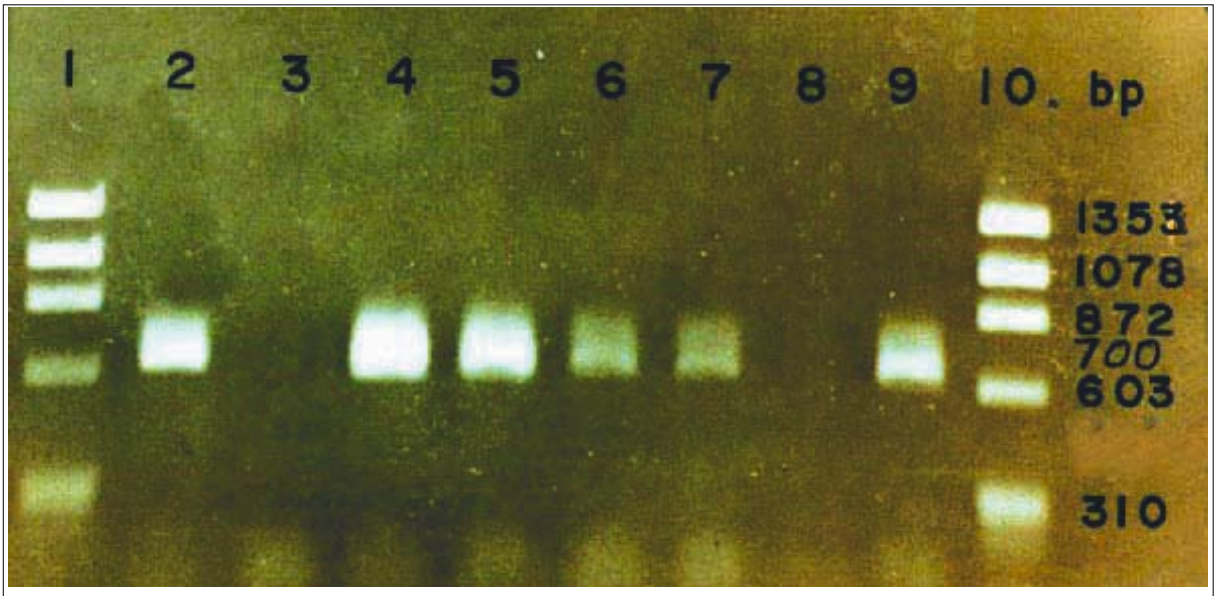

Fig. 2. Specific amplification of invasion plasmid antigen H (*ipa*H) locus DNA by PCR. Amplified products of each reaction were analyzed by electrophoresis through a 0.8%-agarose gel. Lanes: 2, *S. flexneri* M90T used as a positive control (culturable); 3 and 8 negative controls without template; 4 through 7 and 9 non-culturable *S. dysenteriae* type 1 detected from cloth, wood, plastic, aluminum and glass surfaces respectively, five days after inoculation; Lanes 1 and 10, HaeIII digest of ϕx174 replicative form DNA

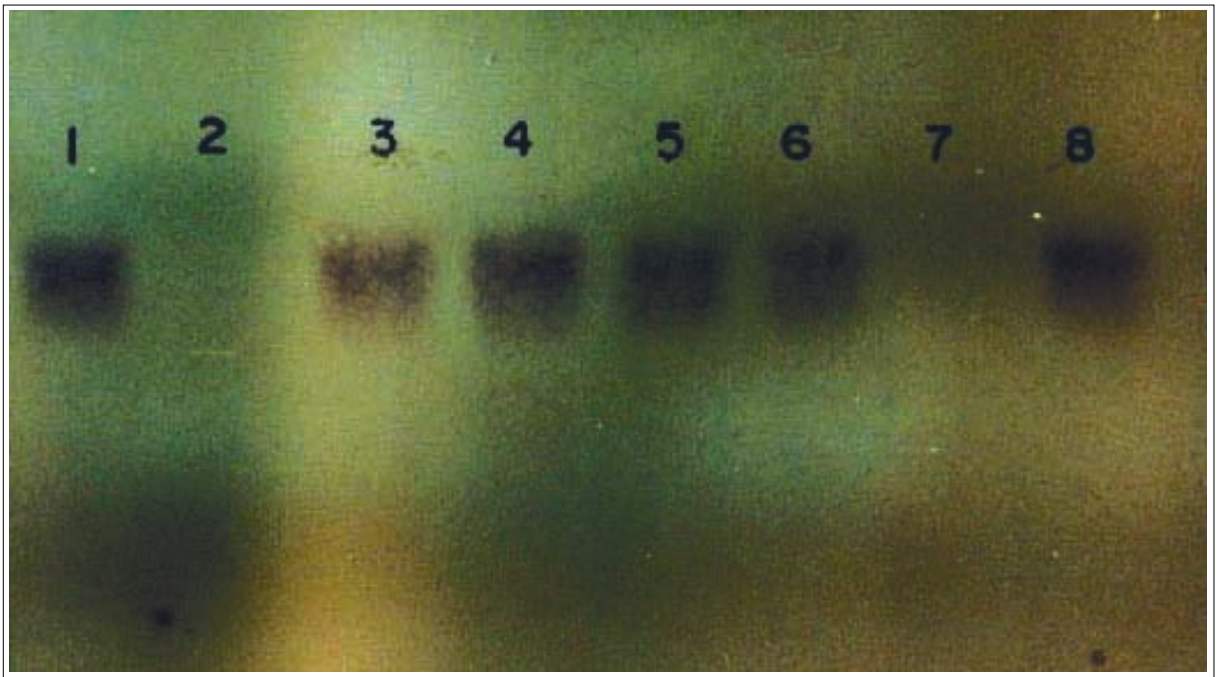

Fig. 3. Hybridization of southern blot of panel 2 (Fig .2) with alkaline phosphatase-labelled *ipa*H probe. Lanes: 1, *S. flexneri* M90T used as positive control (culturable); 2 and 7, negative controls without template; 3 through 6 and 8, non-culturable *S. dysenteriae* type 1 detected from cloth, wood, plastic, aluminum and glass surfaces respectively, 5 days after inoculation

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