



Pathogenic *Vibrio parahaemolyticus* in diarrhoeal patients, fish and aquatic environments and their potential for inter-source transmission

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ARTICLE INFO

Keywords:

Epidemiology
Public health

ABSTRACT

The role of *Vibrio parahaemolyticus* in causing diarrhoeal disease is well known. However, phenotypic and genetic traits of this pathogen isolated from diverse sources have not been investigated in detail. In this study, we have screened samples from diarrhoeal cases (2603), brackish water fish (301) and aquatic environments (115) and identified *V. parahaemolyticus* in 29 (1.1%), 171 (56.8%) and 43 (37.4%) samples, respectively. Incidence of pathogenic *V. parahaemolyticus* with virulence encoding thermostable-direct haemolysin gene (*tdh*) was detected mostly in fishes (19.3%) and waters (15.6%) than clinical samples (1.04%). The pandemic strain marker genes (*toxRS* and *PGS*-sequences) have been detected relatively more in water (6%) and fish (5%) samples than in clinical samples (0.7%). Majority of the *V. parahaemolyticus* isolates from clinical cases and fish samples (26.3%) belonged to classical pandemic serovars (O3:K6). In addition, several newly recognised pandemic serovars have also been identified. Pulsed field-gel electrophoresis (PFGE) analysis showed clonal relatedness (60–85%) of *V. parahaemolyticus* from different sources. The study observation revealed that the brackish water fishes and water bodies may act as a reservoir of pathogenic *V. parahaemolyticus*. Emergence of several new serovars of pandemic *V. parahaemolyticus* signifies the changing phenotypic characteristics of the pathogen.

1. Introduction

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium that remains one of the leading causes of diarrhoea and gastroenteritis [1, 2]. Thermostable-direct haemolysin (TDH) and TDH-related haemolysin (TRH) are considered to be the major virulence factors in this bacterium [1]. Almost all the clinical isolates expressed TDH and/or TRH encoded in the *tdh* and *trh* genes, respectively [1, 3]. These genes are rarely found in environmental *V. parahaemolyticus* isolates [4, 5].

Till 1995, *V. parahaemolyticus* has been implicated with sporadic diarrhoea. With the emergence of unique serovar (O3:K6) in 1996, this organism has gained pandemicity and caused diarrhoeal outbreaks in several countries [6, 7, 8, 9, 10, 11, 12, 13]. The O3:K6 clone (pandemic strain) and its serovariants typically possess *tdh* gene with a distinctive

toxRS operon that encodes a transcriptional regulator. To facilitate identification of pandemic O3:K6, group specific (GS-PCR) and ORF-8 PCRs targeting the *toxRS* operon and filamentous phage f237, respectively have been developed [9, 14]. Besides, PGS-PCR assay directed to detect a 930 bp sequence is used for detection of pandemic strain of this pathogen [15].

Studies conducted in India have reported the prevalence of pandemic *V. parahaemolyticus* strain mainly from hospitalized diarrhoeal cases [16, 17, 18, 19], sewage [20]; salt water fishes, shellfishes and coastal environment [21, 22, 23, 24]; however, no concurrent study has been made to detect the reservoirs of this pathogen. To address this lacuna, we conducted a detailed study using samples collected from different sources in and around Kolkata and also by characterizing the *V. parahaemolyticus* isolates.

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<https://doi.org/10.1016/j.heliyon.2019.e01743>

Received 20 July 2018; Received in revised form 12 February 2019; Accepted 13 May 2019

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2. Materials and methods

2.1. Sampling

Stool specimens from 2603 diarrhoeal cases admitted in the Infectious Diseases Hospital (IDH) and outpatients attending the BC Roy Child Memorial Hospital for children, Kolkata were collected during 2008–2011. During the same period, 301 brackish and freshwater fish from different markets and 115 water samples in and around Kolkata metropolis were collected and screened for the presence of *V. parahaemolyticus*.

2.2. Isolation and identification of *V. parahaemolyticus*

All the samples were screened for *V. parahaemolyticus* by adopting the published cultural and biochemical methods [11, 25]. Briefly, 2–4 loopful of diarrheal stool was pre-enriched in 2 ml of alkaline peptone water (APW) containing 3% NaCl, pH 8.5. About 15 g of gill from each fish was aseptically dissected and homogenized before inoculation in 50 ml of APW. About 100 ml of water sample was filtered aseptically through 0.45 µm membrane and placed in 10 ml of APW and incubated aerobically for 18 hrs at 37 °C.

A loopful of pre-enriched culture was streaked on thiosulfate citrate bile salts sucrose (TCBS) agar (Eiken, Tokyo, Japan) and incubated at 37 °C for 18–24 hrs. Presumptive identification of *V. parahaemolyticus* was made based on typical colony characteristics, i.e., 2–3 mm sized round non-sucrose fermenting green color colonies. Five typical colonies from each sample were selected for biochemical characterization using a multi-test medium in which *V. parahaemolyticus* gave acidic (yellow) butt and alkali (purple) slant (K/A) reaction [25]. *V. parahaemolyticus* isolates were further subjected to PCR assays targeting species-specific *toxR* gene [26], virulence encoding genes (*tdh* and *trh*) [27] and pandemic strain marker genes (*toxRS*, *orf-8* and PGS-sequence [9, 14, 15].

2.3. Reference strains Bacterial strains

Laboratory reference strains Vp-Kx-V138 and VP230 were used as a positive control and *Escherichia coli* K12 strain as a negative control in the PCR assays.

2.4. Molecular characterization of isolates

2.4.1. Bacterial template DNA preparation

A loopful of overnight culture from LB agar with 3% NaCl (LBS) was taken in 1.5 ml microfuge tubes containing 200 µl sterile distilled water and was suspended well using a vortex mixture. This mixture was boiled for 10 min and rapidly cooled on ice. The cell suspensions were centrifuged at 4,000 rpm for 3 min and the supernatant was used as a genomic DNA template. The PCR assay was performed using the Gene Amp PCR system 2700 thermocycler (Applied Bio-systems).

2.4.2. Species-specific and virulence gene (*Vp-toxR*, *tdh*, *trh*) PCR

To detect the *toxR* gene by PCR, primers F: GTCTTCTGACGCAATCGTTG and R: ATACGAGTGGTTGCTGTCATG were used as described previously [26]. All the *toxR* confirmed isolates were further examined for the presence of virulence encoding *tdh* and *trh* genes using the primer pair F: CCAAATACATTTTACTTGG and R: GGTACTAAATGGCTGACATC and primer pair F: GGCTCAAATGGTTAAGCG and R: CATTTCGCTCTCATATGC, respectively [27].

2.4.3. Detection of pandemic marker: [*toxRS* new/GS-PCR; pandemic group-specific PCR (PGS-PCR) and *orf-8*]

V. parahaemolyticus isolated from different sources were further subjected to determine the presence of pandemic marker genes by using the published methods. GS-PCR assay was performed using GS-VP1 (TAATGAGGTAGAAACA) and GS-VP2 (ACGTAACGGGCTACA) primer

pair [9], PGS-PCR assay using F: TTCGTTTCGCGCCACAAC and R: TCGGTGATTATCGCGTCT [15] and *orf-8* gene PCR assay using F: AGTATTGCTGAAGAGTACG and R: CTCGACTTAAACGATCCC primer pairs [14]. All the PCR amplified products were analyzed by gel electrophoresis.

2.5. Serotyping

V. parahaemolyticus isolates were serotyped using somatic (O) and capsular (K) antisera (Denka Seiken, Co. Ltd., Tokyo) [28].

2.6. Pulse field-gel electrophoresis (PFGE)

For clonal identification, the pandemic serovars were examined by PFGE following the PulseNet protocol (CDC, 2009) [29]. Briefly, test isolates were grown on LAS and incubated overnight at 37 °C. The overnight culture was suspended in a cell suspension buffer (CSB) (100 mM Tris, 100 mM EDTA, pH 8.0) and measured the cell density in a spectrophotometer with OD value between 1.3 and 1.5 at 600 nm. Agarose plugs were prepared by mixing equal volume of bacterial suspension with 1% low-melting agarose (Sea-Kem). After solidification, the sized plugs were treated with cell lysis buffer (50 mM Tris, 50 mM EDTA, pH 8.0 and 1% Sarcosyl) followed by proteinase K (20 mg/ml) at 54 °C for 1 hour with constant shaking (150–175 rpm).

The plugs were washed twice with sterile distilled water (pre-heated to 50 °C) under vigorous shaking in a 50 °C water bath for 10–15 min and further washed 4 times with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Agarose plugs were then equilibrated in TE buffer and were placed in 30 µl of 10X H buffer (0.1% BSA, 0.1% Triton X-100) for 45 min. After incubation, plugs were kept overnight in 150 µl reaction mixture consisting 15 µl 10X H buffer, 15 µl 10X BSA, 3 µl *NotI* enzyme (45 units) [Takara, Shuzo Co. Ltd, Japan] and 117 µl sterile distilled water at 37 °C.

PFGE of the *NotI* digested inserts was performed by the contour clamped homogeneous electric field method on a CHEF Mapper system (Bio-Rad, Hercules, CA, USA) with 1% PFGE grade agarose in 0.5X TBE (44.5 mM Boric acid, 1.0 mM EDTA, pH 8.0) for 18 hrs using the *XbaI* digested DNA of *Salmonella enteritidis* serovar Braenderup as the standard size DNA molecular marker. A mini chiller (Bio-Rad) was used to maintain the temperature of the buffer at 14 °C. Run conditions (150 mA current, voltage-6.0V/cm, angle-120°, initial switch time-10 sec, final switch times-35 sec, linear) were generated by the auto algorithm mode of the CHEF Mapper PFGE system by using a 78–390 kb size range. After electrophoresis, the gel was stained with ethidium bromide (1 µg/ml) for 30 min and destained with water for 15 min twice. The DNA bands were visualized and photographed with the BioSpectrum AC Imaging System (USA).

The PFGE image was captured using a Gel Doc XR system (Bio-Rad). Each DNA band was normalized by aligning with the peaks of the *Salmonella enterica* serovar Braenderup (H9182) and analyzed using the BioNumerics software Version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Degree of banding similarity was determined by comparison of the Dice coefficient, and clustering correlation coefficients were calculated by an unweighted pair-group method with arithmetic averages (UPGAMA).

3. Results

3.1. Isolation of pathogenic *V. parahaemolyticus*

The proportion of *V. parahaemolyticus* identified from diarrhoeal patients, fishes and water samples are shown in Table 1. In the species-specific PCR (*Vp-toxR*), all the isolates from clinical stool specimens were found to be positive for *V. parahaemolyticus*. Similarly, 171 of 210 isolates from retail fishes and 43 of 53 isolates from water samples were identified as *V. parahaemolyticus*, confirming the occurrence of this pathogen in 56.8% and 37.4% of retail fish and water samples,

Table 1Isolation and molecular characterization of *V. parahaemolyticus* isolates from clinical diarrhea, retail fish and water.

Sample Source (no.)	Identification (%)		Virulence encoding gene (%)		Pandemic strain marker (%)		
	Culture	Vp-toxR	tdh	trh	ORF-8	GS-PCR	PGS-PCR
Clinical diarrhea (2603)	29 (1.1)	29 (1.1)	27 (1.04)	6^a (0.2)	13 (0.5)	19 (0.7)	19 (0.7)
Retail fish (301)	210 (69.7)	171 (56.8)	58 (19.3)			12 (4)	15 (5)
Razor fish (64)	53 (82.8)	42 (65.6)	12 (18.7)			1 (1.5)	2 (3.1)
(<i>Aeoliscustrigatus</i>)							
Sardine (30)	24 (80)	23 (76.6)	5 (16.7)			2 (6.7)	2 (6.7)
(<i>Sardinops neo pilchardus</i>)							
Mackerel (25)	19 (76)	19 (76)	4 (16)			1 (4)	2 (8)
(<i>Rastrelligerkanagurta</i>)							
Conch fish (74)	49 (66.2)	34 (46)	19 (25.6)			5 (6.7)	6 (8.1)
(<i>Dasyatisbennettii</i>)							
Phasa fish (45)	33 (73.3)	27 (60)	15 (33.3)			2 (4.4)	2 (4.4)
(<i>Setipinnaphasa</i>)							
Rohu (45)	17 (37.8)	11 (24.4)	1 (2.2)				
(<i>Labeorohita</i>)							
Hilsafish (<i>Hilsailisha</i>) (18)	15 (83.3)	15 (83.3)	2 (11.1)			1 (5.6)	1 (5.6)
Water (115)	53 (46.1)	43 (37.4)	18 (15.6)	2 (1.7)	1 (0.9)	6 (5.2)	7 (6)
River (29)	17	12 (41.38)	5 (17.2)			1 (3.4)	1 (3.4)
i) Ganga (21)	9	4 (19)	3				
ii) Ischhamati (8)	8	8 (100)	2 (25)			1 (12.5)	1 (12.5)
Estuary (54)	36	31 (57.4)	13 (24)	2 (3.7)	1 (1.9)	5 (9.2)	6 (11.1)
i) Kakdweep (7)	6	6 (85.7)	5 (71.4)	1 (14.3)	1 (14.3)	1 (14.3)	2 (28.6)
ii) Canning (7)	7	7 (100)	1 (14.3)				
iii) Dhamakhali (34)	23	18 (53)	7 (20.6)	1 (3)		4 (11.8)	4 (11.8)
iv) Diamond harbour (6)							
Fresh water (25)							
i) Pond (16)							
ii) Jheel (9)							
iii) Seedling pond (7)							

^a One clinical isolate (*tdh*[−]/*trh*⁺) was positive in GS and PGS-PCR.

respectively (Table 1).

Out of the total 29 isolates from clinical diarrhoeal cases, 27 were found positive for presence of *tdh* and 5 *tdh*⁺ isolates were also positive for *trh* (Table 1). Besides, one isolate was positive for *trh* only. Overall, the detection rate of *V. parahaemolyticus* in diarrhoeal cases was very less (1.1%, 28/2603). From fish and water samples, *tdh* harbouring *V. parahaemolyticus* was detected in 19.3% (58/301) and 15.6% (18/115), respectively. Two isolates (1.7%) from water sources were positive for both *tdh* and *trh* (Table 1).

Among retail brackish water fishes, *tdh* positive *V. parahaemolyticus* was detected in 33.3% of Phasa fishes (*Setipinnaphasa*) followed by Conch fishes (*Dasyatisbennettii*) (25.6%), Razor fish (*Aeoliscustrigatus*) (18.7%), Sardine (*Sardinopsneopilchardus*) (16.7%) and Meckerel (*Rastrelligerkanagurta*) (16%). Fresh water fishes like Rohu (*Labeorohita*) and Hilsa (*Hilsailisha*) were also found to harbour *tdh* positive *V. parahaemolyticus* in 2.2% and 11.1% of samples, respectively (Table 1). The pathogenic *V. parahaemolyticus* was identified in river (17.2%) (5/29) and estuarine waters (24%) (13/54). However, no isolation of *V. parahaemolyticus* was made from fresh water samples viz. pond, Jheel and seedling pond (Table 1).

3.2. Occurrence of *V. parahaemolyticus* pandemic strain

Results of GS-PCR revealed that 0.7% of diarrhoeal cases, 4% of retail fishes and 5.2% of water samples contain epidemic strain of *V. parahaemolyticus*. The PGS-PCR assay gave almost similar results (Table 1). However, *orf-8* was very less, i.e., 0.5% in clinical and 0.9% in water isolates and none of fish samples (Table 1).

In the GS-PCR assay, pandemic strain of *V. parahaemolyticus* was recovered from 6.7% each of Conch and Sardine fishes; 5.6% of Hilsa; 4.4% of Phasa; 4% of Mackerel and 1.5% of Razor fishes. Detection of the pandemic isolates by PGS-PCR assay was slightly higher than the GS-PCR (Table 1). As detected in the GS-PCR assay, the pandemic *V. parahaemolyticus* was recovered in water samples from the Ischhamati river (12.5%) and estuarine waters of Kakdweep (14.3%) and

Dhamakhali (11.8%) (Table 1). Like fish samples, isolation of pandemic strain from water samples by PGS-PCR was slightly more than the GS-PCR (Table 1).

3.3. Characteristics of pandemic strain

Analysis of results from clinical isolates revealed that out of a total 19 isolates (*toxRS*⁺ & PGS Sequence⁺), 17 belonged to the established pandemic serovars (O3:K6; O1:KUT; O1:K25; O3:KUT); however, two (2) isolates belonged to O2:K4 and O8:K21 that were new in the list of pandemic serovars.

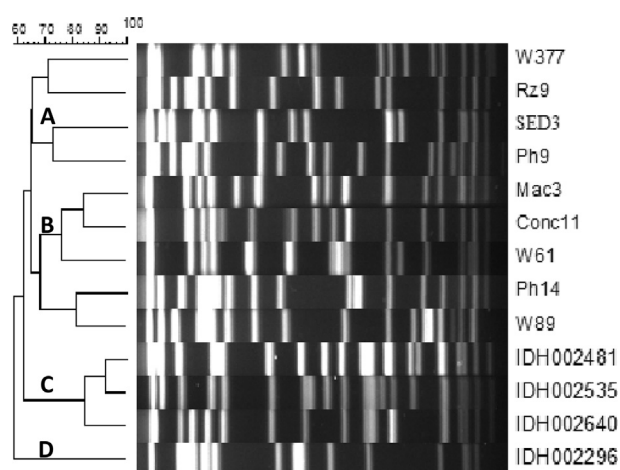
Out of 12 GS/PGS-PCR positive isolates from fishes, pandemic serovars viz. O1:K33, O1:KUT, O5:KUT, O8:KUT, O10:KUT recorded in 7 isolates. Besides, serovars O1:K28; O1:K34; O3:K31; were identified as novel pandemic serovars (Table 2). From water samples, pandemic serovars viz. O4:K12; O5:KUT and OUT:KUT were identified in 4 isolates. In addition, serovar OUT:K33 was recovered as novel pandemic serovar in 2 isolates. Pandemic serovar O5:KUT and new pandemic serovar O8:K21 were found among the *orf-8* positive isolates from water and clinical and samples (Table 2).

3.4. Molecular typing

PFGE of the pandemic *V. parahaemolyticus* was performed to show the extent of clonal relationship between isolates from different sources. A total of 13 representative isolates (GS-PCR⁺ and PGS-PCR⁺) from human (4) fish (6) and water (3) isolates were selected and examined. Dendrogram of PFGE analysis showed three major clusters (A-C, Fig. 1). In cluster 'C', three clinical isolates (IDH 002481; IDH 002535 and IDH 002640) were grouped; of these, IDH 002481 and IDH 002535 belonged to a single clade with >90% similarity and also exhibited >80% relatedness with another human isolate (IDH 002640) in the same cluster. These clinical isolates had ~62% similarity with fish and water isolates in clusters 'A' and 'B', respectively. Further, cluster 'A' and 'B' had approximately 65% similarity. The other clinical isolate (IDH002296)

Table 2Molecular characteristics of different serovars of *V. parahaemolyticus* from clinical diarrhoea, fish and water sources.

Serovar	Source							
	Human (n = 28)		Fish (n = 58)			Water (n = 18)		
	<i>tdh/trh</i>	<i>toxRS</i> &PGS-PCR	<i>tdh</i>	<i>toxRS</i>	PGS-PCR	<i>tdh/trh</i>	<i>toxRS</i>	PGS-PCR
O1:K25	10	9 (6*)						
O1:K28			2	2	2			
O1:K33			2	1	1			
O1:K34			1	1	1			
O1:KUT	2	1 (*)	4	2	3			
O2:K4	1	1						
O2:K28						1		
O2:KUT			2					
O3:K6	5	5 (*4)						
O3:K31			4	2	1			
O3:KUT	4	2 (*1)	3		1			
O4:K8	1							
O4:K9			1					
O4:K12						2	1	1
O4:K25	1							
O4:K42			1					
O4:K63	1							
O4:KUT			4		1			
O5:K15			1					
O5:K17			5					
O5:KUT			8	2	3	7	2 (*1)	2
O6:K15			1					
O7:K19			1					
O8:K1			1					
O8:K21	1	1 (*)						
O8:KUT	1		1	1	1			
O10:KUT			8	1	1	2		
O11:K28			1					
O11:KUT			1					
OUT:K28			2					
OUT:K33						2	2	2
OUT:K55			1					
OUT:KUT			2			4	1	2
OUT:K15			1					
Total	27	19	58	12	15	18	6	7

Asterisk (*) in parentheses indicates the number of isolate positive for *orf-8* gene. One clinical isolate with *tdh*[−]/*trh*⁺ was typed as O5:KUT.**Fig. 1.** Phylogenetic tree (dendrogram) of *Vibrio parahaemolyticus* isolates from human, fish and water using the software, Bio-Numerics (Applied Maths, Belgium). The similarity scale is on the top of figure. IDH: Human clinical isolates, RZ, SED, Ph, Mac, Conc: Isolates from fishes, W = Isolates from water, RZ- Razor; Con- Conch; Ph-Phasa; SRD- Sardine, Mac-Mackerel, W-water.

that discretely belonged to cluster 'D' showed ~60% relatedness with other clinical (n = 3), fish (n = 6) and water (n = 3) isolates of clusters 'A' to 'C' (Fig. 1).

The isolates from water (W377) and Razor fish (Rz9) belonged to the same clade in cluster 'A' with 70% genetic relatedness. Similarly, other

two fish isolates from Sardine (SED3) and Phasa (Ph9) were grouped in the same clade with 70% similarity to each other and showed 65% similarity to Razor fish and water isolates (W377 and Rz9) belonged to other clade in cluster 'A'. In Cluster 'B' isolates from Mackerel (Mac3) and Conch fish (Conc11) were grouped in the same clade with 85% similarity to each other. Further, these two isolates were related (~75%) to one isolate (W61) from water sample. Likewise, one isolate each from fish Phasa (Ph14) and water (W89) belonged to one clade in cluster 'B' showed ~80% similarity and were related (70%) to the other two types of fish isolates (Mac3 and Conc11) and one water isolate (W61).

4. Discussion

Analogous to the previous studies conducted in Indian context [7, 8, 16, 17, 18, 19, 20], we found that association of *V. parahaemolyticus* with diarrhoeal cases was less. Considering environmental reservoirs of *V. parahaemolyticus*, our observation was analogous to other studies [17, 30]. Epidemiologically, this aspect is important because a larger number of coastal and inland populations are engaged in aquaculture for their livelihood and possibility of infection is high. Since *V. parahaemolyticus* is autochthonous to coastal waters, many of the brackish water fishes carry this organism. Occurrence of *V. parahaemolyticus* in fresh water fish like Rohu may be due to cross contamination with other marine fishes sold in the market. The reason for the existence of *V. parahaemolyticus* in Hilsa fish may be due to their anadromous migratory nature.

The performance of the GS-PCR and PGS-PCR seems to vary depending on the nature of the sample [11, 12, 24]. We found that it would be prudent to adopt both the PCR assays to detect the pandemic

strain of *V. parahaemolyticus*, particularly from the environmental sources. However, the *orf-8* PCR has identified limited number of pandemic *V. parahaemolyticus* isolates harbouring the filamentous phage (f237).

Majority of the clinical isolates of *V. parahaemolyticus* belonged to the typical pandemic serovars. However, the other new serovars such as O1:K28; O1:K34; O2:K4; O3:K31; O8:K21 and OUT:K33 identified in this study had the characteristics of pandemic strain. This indicates the rapid phenotypic and genetic changes among *V. parahaemolyticus*. Several studies correlate El Nino event responsible for such changes in the epidemiology of *V. parahaemolyticus* [31, 32]. It is pertinent to mention that during 1995 and 1996, only the O3:K6 was identified as pandemic serovar; however, with the passage of time, several serovars have been recognized in this category [10, 33]. Since the overall genetic relationship was almost similar among different isolates from human, fish and water bodies, it is likely that food animals and water bodies may act as reservoirs of pandemic strains of *V. parahaemolyticus*.

5. Conclusion

Pandemic strain of *V. parahaemolyticus* mostly detected in retail brackish water fishes and aquatic bodies with several new serovars. PFGE analysis has shown the genetic relatedness of *V. parahaemolyticus* isolates from different sources, signifying potential reservoirs of human infection.

Declarations

Author contribution statement

Sailen Guin, Murugan Saravanan, Prasad Anjay, Goutam Chowdhury, Pazhani Gururaja Perumal: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Thandavarayan Ramamurthy. Suresh Chandra Das: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

This work was supported by the Indian Council of Medical Research (ICMR), New Delhi, India under extra mural research grant (IRIS ID 2003 02200).

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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