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Occurrence of *Enterobacteriaceae* with Serological Cross Reactivity towards *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae* in Food

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MF and MMA planned the study. Authors MF and MMR performed the experiments. Authors MMH and MMA supervised the study. Author MF wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was conducted to determine the occurrence of serologically cross-reactive enterobacteriaceae in food samples.
Place and Duration of Study: This study was conducted in Industrial Microbiology Laboratory, IFST, BCSIR, Dhaka, Bangladesh during January-November, 2013.
Methodology: 14 Enterobacteriaceae was isolated from food samples and identified. Serological reaction was determined by slide agglutination method.
Results: Among 14 isolates, 6 were identified to be *Cronobacter* spp., 3 to be *Shigella* spp., 2 to be *Enterobacter* spp., 2 to be *Escherichia coli* and 1 as *Klebsiella* spp. Three of the isolates belonging to *Cronobacter* showed extremely strong cross-reactivity with *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae*. Other isolates gave relatively weak but significant cross-reactions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell surface protein also showed similar bands in the isolates as type strains of *Salmonella, Shigella* and *Vibrio cholerae*.

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Conclusion: The results indicate that *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae* surface antigens are shared by *Enterobacteriaceae* present in food.

Keywords: Enterobacteriaceae; serological; cross-reactivity; food.

1. INTRODUCTION

The *Enterobacteriaceae* is a family of Gram-negative, non-spore-forming bacteria [1]. Many species of Enterobacteriaceae are members of the normal intestinal flora of animals and humans. This family includes a number of important foodborne pathogens such as *Salmonella, Yersinia enterocolitica, E. coli* (including *E. coli* O157:H7), *Shigella* spp. and *Cronobacter* spp. Other members of the family (such as *Klebsiella* spp., *Serratia* spp., *Citrobacter* spp.) are regarded as opportunistic pathogens [2]. Enterobacteriaceae are of great concern in the food industry due to the fact that they pose a severe threat to safety. Moreover, being zoonotic pathogens they negatively affect human health and are able to cause a number of nosocomial infections [1]. Enterobacteriaceae are also of concern in food safety as due to their ubiquitous nature it is very difficult to prevent their incorporation in food chain [3]. Gram-negative bacteria of the Enterobacteriaceae family are known to cause of urinary tract infections (UTIs), bloodstream infections, hospital- and healthcare-associated pneumonias, and various intra-abdominal infections [4].

The Gram negative bacterial pathogens have complex antigenic structures expressing several protein and lipopolysaccharide (LPS) components on the surface that constitute to their serological reactivity [5]. LPS is a vital part of the outer membrane of Gram- negative bacteria and is a major virulence factor. Serological characterization based on the antigenic determinants of antigens expressed on the bacterial cell surface is of importance in the diagnosis of bacterial species [6]. Bacterial strains carrying identical or similar antigenic components on the cell surface might also react with antibodies produced against other strains [7]. Environmental factors induces changes in LPS structure of bacteria [8]. Moreover, co-existence of bacteria species in complex microbial communities within the environment may cause shifting of LPS structure among bacteria. This results in cross-similarity in LPS leading to cross reactivity during serological assays [8]. Non-pathogenic or opportunistic pathogenic bacteria having serological cross-reactivity with pathogenic bacteria may acquire virulent antigenic determinants and be able to cause diseases [9].

The aim of this study was to isolate *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae* that are well-known human pathogens from food samples and determine their serological cross-reactivity with commercial anti sera.

2. MATERIALS AND METHODS

2.1 Food Sample Collection

Fifty four (54) different food products from different manufacturers were purchased from retail stores across Dhaka, Bangladesh. The samples consisted of 15 milk powder, 6 horlicks, 6 honey, 6 chutney, 6 chocolates, 10 biscuits and 5 spices.

2.2 Isolation

The procedure of FDA for detection, isolation and identification of members belonging to the Enterobacteriaceaein food samples was utilized in the analysis of samples. All of the food samples were diluted with buffered peptone water (BPW) in 1:10 (10g of samples/ 90ml BPW) ratio for pre-enrichment and incubated for 18-24 hrs at 37ºC. The pre-enriched samples were added to Enterobacteriaceae enrichment (EE) broth (Oxoid Ltd., UK) in a 1:10 ratio. The EE broth contains bile salt and brilliant green which suppress the growth of bacteria that do not belong to the family Enterobacteriaceae and samples were incubated at 37ºC for 18-24 hrs. An aliquot of 100µL from each EE broth sample was spread-plated on violet red bile glucose (VRBG) agar. Another loopful of the suspension was streaked on VRBG agar. The plates were incubated for 18-24 hrs at 37ºC. Characteristic red or pink colonies on VRBG agar were sub cultured onto MacConkey agar (HiMedia, India), Xylose Lysine Deoxycholate (XLD) agar (Oxoid, UK) and *Cronobacter sakazakii* agar (HiMedia, India) for selective isolation of different members of Enterobacteriaceae. All the isolates were further streaked on Tryptone soy agar (TSA) to observe pigment production.

2.3 Identification

Gram Staining, oxidase test, catalase test, citrate utilization test, methyl red, Voges-Proskauer, Kligler's iron agar (KIA), nitrate reduction test, arginine decarboxylase, gelatin hydrolysis and indole production, production of gas from glucose at 37ºC were used for biochemical identification of isolates. Xylose, trehelose, ducitol, arabinose, salicin, mannitol, sucrose, lactose, sorbitol, maltose and esculin were used in the carbohydrate fermentation assay. Identification was inferred from Bergey's manual of systematic bacteriology [10].

2.4 Agglutination Test for Strains

Commercially available agglutinating serum (Remel, UK) such as *Salmonella* 2-0, *Salmonella* 9-0, *Salmonella* H, *Salmonella typhi* O-Group D somatic antigen, *Salmonella paratyphi* A-O Group A somatic antigen, *Salmonella* polyvalent O group A-S, *Shigella boydii* polyvalent 3(12-13), *Shigella boydii* polyvalent 1(1-6), *Shigella boydii* polyvalent 2(7-11), *Shigella sonnei* phase 1 & 2, *Shigella flexneri* polyvalent (1-6, x & y), *Vibrio cholerae* O1 polyvalent, *Vibrio cholera*e inaba, *Vibrio cholera*e ogawa were used for serological agglutination reactions of strains. 25µL of sterile 0.85% (w/v) saline was placed on a glass slide and bacterial colonies were emulsified in it to get a homogenous milky-white suspension. Then 10µL of antisera was added to the bacterial suspension and the slide was rotated and macroscopic agglutination was observed within one minute.

2.5 SDS-PAGE Analysis of Surface Proteins

Enterobacteriaceae isolates were cultivated into tryptone soya broth (TSB) and cell pellets were collected by centrifugation (12,000rpm). The pellet was then washed twice with phosphate buffer saline (PBS, pH 7.4), resuspended in 1mL of extraction buffer (10% Glycerol, 2% SDS, 0.05M Tris, pH 6.8) and then boiled for 3 minutes at 100ºC. The supernatant was then separated, filtered through 0.45µm milipore membrane and stored at -20ºC [11]. This whole cell protein extract was mixed with an equal volume of concentrated sample loading buffer and was heated at 80ºC for 10 minutes. The processed samples were then loaded onto a 12% (w/v) SDS-polyacrylamide gel as previously described by Laemmli [12]. Electrophoresis was carried out at 30mA until the tracker dye (Bromophenol blue) reached the bottom of the gel. Gels were stained for protein with Coomassie Brilliant Blue (CBB) destaining with a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) methanol. Molecular weights were estimated using SDS-PAGE protein molecular weight pre-stained markers (Promega, USA).

3. RESULTS

3.1 Isolation and Identification of Isolates

All the fourteen isolates produced characteristic red or pink colonies on VRVG agar (Oxoid, UK). Two isolates (M1 & S6) produced water like yellow pigmentation and twelve isolates (M2, M4, M5, M6, M10, H1, H2, H5, N1, C2, B5 & B9) produced yellow pigmentation on TSA respectively. On the basis of Gram staining, biochemical characters and carbohydrate utilization test results, isolates were identified as presumptive *Cronobacter* spp. (M1, M2, M10, H5, B5& S6); *Klebsiella* spp. (M4), *Enterobacter* spp. (M5 & M6), *Shigella* spp. (H1, H2 and N1) and *Escherichiacoli* (C2 & B9) according to Bergey's manual of systematic bacteriology [10].

3.2 Serological Cross Reactivity

The isolates showed significant serological cross reactivity with different serotypes of *Salmonella*, *Shigella* and *Vibrio cholerae*. Results of the serological cross reactivity tests are shown in Table 1. Among 14 bacterial isolates, six *Cronobacter* isolates (M1, M2, M10, H5, B5 & S6) showed cross-reactivity with six different serotypes of *Salmonella*, five different serotypes of *Shigella* and in eachcase, highly type-specific. One of the isolates belonging to *Klebsiella* showed strong cross-reactivity with *Salmonella* polyvalent O group A-S,*Shigella flexneri* polyvalent (1-6, x & y) &*Vibrio cholera* inaba. Two *Enterobacter* isolates (M5 & M6) showed cross-reactivity with different serotypes of *Salmonella*, five different serotypes of *Shigella*, three serotypes of *Vibrio cholera*. Three *Shigella* isolates (H1, H2 & N1) showed cross-reactivity with single serotypes of *Salmonella* polyvalent O group A-S and different serotypes of *Shigella boydii.*Two *E. coli* isolates (C2 & B9) showed cross-reactivity with serotype of *Salmonella* polyvalent O group A-S and *Shigella flexneri* polyvalent (1-6, x & y).

A large proportion (78.5%) enterobacteriaceae isolate pose cross-reactivity with *Salmonella* polyvalent O group A-S. 57.15% isolate showed cross-reactivity with *Salmonella* 2-O, *Salmonella* H, *Shigella boydii* polyvalent 3 (12-13) and *Shigella boydii* polyvalent 1 (1-6); 64.2% with *Shigella flexneri* polyvalent (1-6, x & y) and Vibrio cholerae O1 polyvalent; 50% with *Salmonella paratyphi* A-O Group A somatic antigen and *Vibrio cholerae* inaba; 42.9% with *Salmonella typhi* O-Group D somatic antigen and *Shigella boydii* polyvalent 2 (7-11); 35.7% with *Shigella sonnei* phase 1 & 2 and 28.5% with *Salmonella* 9-0. No isolate was found to cross-react with *Vibrio cholerae* ogawa (Fig. 1).

Three (3) isolates (M2, M10, H5) showed cross-reactivity to more than 10 different antibodies used in this study, 6 isolates (M1, M5, H2, N1, C2, B5) were reactive to 5-9 antibody and 5 isolates (M4, M6, H1, B5, S6) were reactive to less than 5 antibody. Cronobacter isolate H5 were reactive to 12 antibody and isolate M2 & M10 were reactive to 11 antibodies. High cross-reaction of *Cronobacter* isolates is of particular concern as it is considered as potential threat to children due to its contamination of powdered infant formula [13].

3.3 SDS-PAGE of Cell Surface Proteins

SDS-PAGE of cell surface proteins of the isolates confirms similarity between the isolates with *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae* (Fig. 2). Similar protein bands between isolate and reference organism indicates serological cross-reactivity. SDS-PAGE of cell surface proteins of the isolates also demonstrated serological cross-reactivity. The isolates showed to possess some bands similar to the strains of *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae*. Though banding patterns and number of bands vary among the isolates (Fig. 2), all the isolates showed bands similar to type strains in SDS-PAGE corresponding to the slide-agglutination reactions.

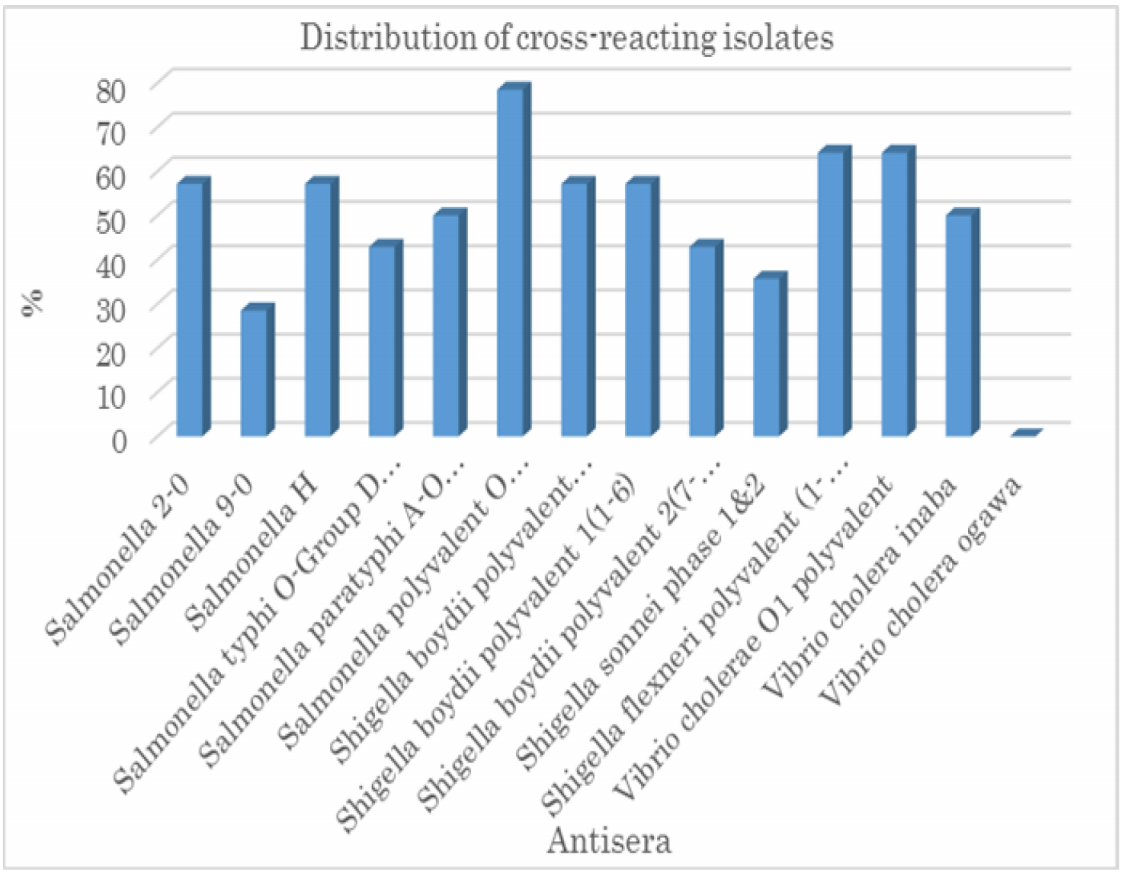

Fig. 1. Distribution of cross-reacting isolates

Table 1. Different antisera agglutination test for isolates

Isolates	1 (Sal)	2 (Sal)	3 (Sal)	4 (Sal)	5 (Sal)	6 (Sal)	7 (Sh)	8 (Sh)	9 (Sh)	10 (Sh)	11 (Sh)	12 (Vc)	13 (Vc)	14 (Vc)
Cronobacter (M1)	+	-	+	-	-	+	+	+	+	-	+	-	-	-
Cronobacter (M2)	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Klebsiella (M4)	-	-	-	-	-	+	-	-	-	-	+	-	+	-
Enterobacter (M5)	+	-	+	+	+	-	+	+	-	+	-	+	-	-
Enterobacter (M6)	-	-	-	+	-	+	-	-	-	-	+	-	+	-
Cronobacter (M10)	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Shigella (H1)	-	-	-	-	-	+	+	-	+	-	-	-	-	-
Shigella (H2)	+	-	+	-	-	+	+	-	+	-	-	-	-	-
Cronobacter (H5)	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Shigella (N1)	-	-	-	+	-	+	+	+	+	-	-	-	-	-
Escherichia coli (C2)	+	-	+	-	+	+	-	+	+	+	+	-	-	-
Cronobacter (B5)	+	-	-	-	+	-	-	-	-	-	+	-	-	-
Escherichia coli (B9)	-	+	+	-	-	+	-	+	+	-	+	-	-	-
Cronobacter (S6)	-	-	-	-	+	-	-	-	-	-	-	-	-	-

(Sal indicates Salmonella; Sh indicates Shigella and Vc indicates Vibrio cholerae) (1. Salmonella 2-0, 2. Salmonella 9-0, 3.Salmonella H, 4. Salmonella typhi O-Group D somatic antigen, 5. Salmonella paratyphi A-O Group A somatic antigen, 6. Salmonella polyvalent O group A-S, 7. Shigella boydii polyvalent 3(12-13), 8. Shigella boydii polyvalent 1(1-6), 9. Shigella boydii polyvalent 2(7-11), 10. Shigella sonnei phase 1 & 2, 11. Shigella flexneri polyvalent (1-6, x & y), 12. Vibrio cholerae O1 polyvalent, 13. Vibrio cholerae inaba, 14. Vibrio cholerae ogawa)

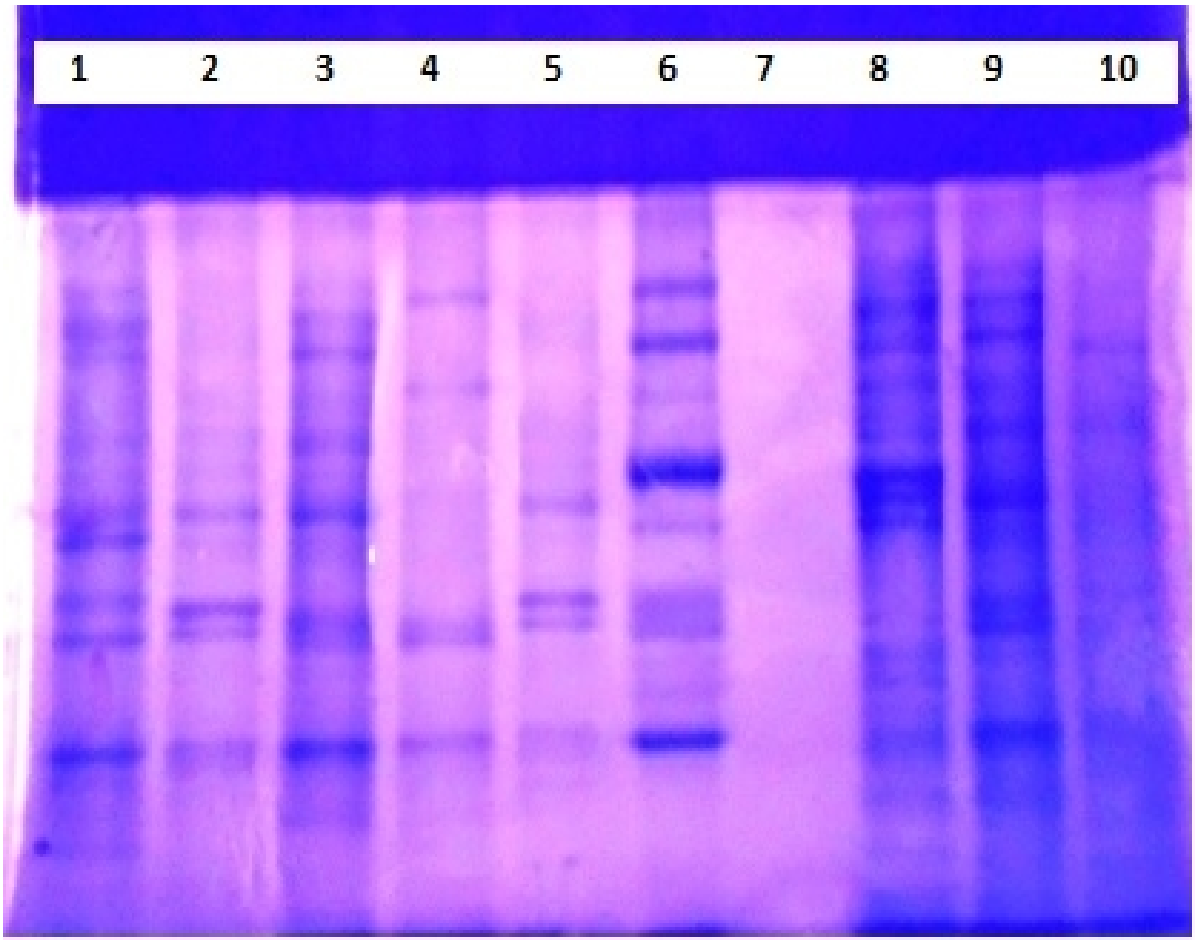

Fig. 2. Comparison of surface proteins of the isolates (Lane 1: M2; Lane 2: M4; Lane 3: M10; Lane 4: H1; Lane 5: M6; Lane 6: C2; Lane 7: S6; Lane 8: *Salmonella* spp. (Environmental isolate); Lane 9: *Shigella flexnerii* (Environmental isolate); Lane 10: *Vibrio cholerae* (Environmental isolate)

4. DISCUSSION

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria and serological heterogeneity is defined by the diversity in the structure of LPS at the cell-surface [14]. The composition of the LPS of bacterial cell surface is influenced by growth and environmental conditions. Environmental conditions during growth have been shown to affect the composition of the LPS wall of Gram negative Enterobacteriaceae [15]. Antigenic determinants expressed on the bacterial cell surface are of importance in the serological characterization and microbiological identification. The bacterial strains carrying these identical or similar antigenic epitopes might react with antibodies produced against other strains [16]. Moreover, non-pathogenic bacteria with cross-reactive antigens of pathogenic ones may cause infection in their hosts [17].

Fourteen Enterobacteriaceae strains were isolated from the food samples and identified biochemically to genus level. Six were identified as *Cronobacter* spp., 1 as *Klebsiella* spp., 2 as *Enterobacter* spp., 2 as *E. coli* and 3 as *Shigella* spp. Many studies have also reported the presence of enterobacteriaceae in different types of foods in Bangladesh [2,3,18].

Cross-reaction against all the commercial antisera (except *Vibrio cholerae* ogawa) was observed. Most of the isolates showed cross reactivity against multiple antisera. Serological cross-reactivity of members of enterobacteriaceae and other Gram negative bacteria have been reported by many researchers [5,6,7,9,19]. Rice et al. [20] reported

serological cross-reaction of different species of *Escherichia* genus with *Escherichia coli* O157. *Aeromonas trota* isolated from water has showed cross-reactivity with *Vibrio cholerae* O139 [19]. Serological cross-reaction between *Escherichia coli* O157:H7, *Citrobacter freundii* and *Citrobacter sedlakii* has also been reported previously [7]. Rahman et al. [8] reported that environmental isolates belonging to the genus *Enterobacter, Escherichia, Stenotrophomonus* and *Aerococcus* revealed cross-reactivity with *Shigella* spp.-specific antisera. Rabbi et al. [9] reported cross-reaction between *Vibrio cholerae* O1, *Shigella flexneri* 2a and *Haemophilus influenzae* B. Kwinkowski et al. [6] reported cross-reactivity of *Shewanella fidelis* with *Proteus*. Interestingly, all the isolates for which cross-reactivity have been reported were either from clinical or environmental sources.There is currently very little documented information on the occurrence of Enterobacteriaceae in food that show cross-reactivity with antisera designed to identify other species. The findings of this study provide preliminary evidence of presence of serological cross-reactivity among enterobacteriaceae with pathogenic micro-organisms in food in Bangladesh.

5. CONCLUSION

Occurrence of serologically cross-reacting Enterobacteriaceae in food samples is alarming and warrants us about the necessity of increased monitoring of hygiene during production and packaging of food products.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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