Epidemiol. Infect. (2015), **143**, 94–103. © Cambridge University Press 2014
doi:10.1017/S0950268814000351

Sorbitol non-fermenting shiga toxin-producing *Escherichia coli* in cattle on smallholdings

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Received 1 September 2013; Final revision 20 January 2014; Accepted 31 January 2014; first published online 27 February 2014

SUMMARY

We investigated faecal samples collected from the rectum of 518 cattle on 371 randomly selected smallholdings in Bangladesh for the presence of sorbitol non-fermenting (SN-F) shiga toxin-producing *Escherichia coli* (STEC). The SN-F isolates were tested for the presence of *rfb* O157, *stx1, stx2, eae* and *hlyA* genes by polymerase chain reaction (PCR). Seven SN-F isolates lacking these genes were profiled by pulsed-field gel electrophoresis (PFGE) to verify their clonality. SN-F *E. coli* was identified in 44 [8·5%, 95% confidence interval (CI) 6·4–11·2] samples; of these, 28 (5·4%, 95% CI 3·8–7·7) had shiga toxin-producing strains, although only two carried the *rfb* O157 gene. Thirteen isolates carried the *hlyA* gene while 18 harboured the *eae* gene. Based on PFGE, six pulsotypes were observed among the seven isolates that had no virulence genes. To the best of our knowledge this is the first report on shiga toxin-producing *E. coli* from direct rectal faecal samples of cattle on smallholdings.

Key words: Cattle, *E. coli*, shiga toxin, smallholdings.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is one of the dominant foodborne zoonotic pathogens worldwide [1, 2]. The pathogenic potential of STEC is attributable to shiga toxins 1 and 2, encoded by *stx1* and *stx2* genes [3]. In STEC the significance of the O157 serogroup in causing haemorrhagic colitis and haemolytic uraemic syndrome (HUS) in humans is well established [4, 5]. Livestock, especially cattle, are natural reservoirs of STEC where they colonize without causing clinical signs [6]. Reports, mostly on STEC O157, are available on cattle reared in organized farms [7, 8]. There are also a few reports on STEC from milk and pooled faecal samples from household cattle in Kenya and Brazil [9, 10]. However, to the best of our knowledge, the carriage intensity of STEC in cattle on smallholdings, based on direct faecal samples from the rectum, and its impact on public health have not been studied before in any developing countries, including Bangladesh.

Selective media have been developed for identifying STEC O157 serogroup [11]. The most common is cefixime and potassium tellurite-supplemented sorbitol MacConkey agar (CT-SMAC) [11–15]. The *E. coli* O157 serogroup do not ferment sorbitol, and consequently grow as colourless colonies following 24 h incubation, unlike typical *E. coli* [16]. However, some sorbitol non-fermenting (SN-F) non-O157 strains have been reported [17]. Intimin, an outer membrane protein, encoded by the *eae* gene, is a virulence-associated factor that causes attaching and effacing lesions in the intestinal mucosa [18].

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Another factor contributing to STEC virulence is enterohaemolysin, a pore-forming cytolysin encoded by a plasmid-borne gene designateded *hlyA* [19]. The presence of both *stx1* and *stx2* genes and the *eae* gene is a potentially highly virulent combination [20]. Molecular characterization of STEC to identify their diversity by the distribution of the two shiga toxin-producing genes – *stx1* and *stx2* – in association with other key virulent genes, such as *eae* and *hlyA* circulating in cattle on smallholdings are important in order to predict the potential threats of STEC to public health, and to suggest approaches to mitigate the threats at source. Based on the distributions of key virulent genes, mentioned above, we describe herein the molecular characteristics of STEC from cattle on smallholdings in Bangladesh by investigating the SN-F bacteria growing on CT-SMAC agar from faecal samples collected directly from the rectum.

Fig. 1 [*colour online*]. Secondary homogeneous growth of sorbitol non-fermenting (SN-F) bacterial colonies on cefixime-potassium tellurite sorbitol MacConkey (CT-SMAC) agar, plated from the culture in tryptic soy broth obtained from multiple ($\geqslant 5$) SN-F colonies inoculated into it from the primary SN-F bacterial colonies developed along with sorbitol-fermenting colonies and others on CT-SMAC agar from a rectal swab collected from a bovine animal on a smallholding in Bangladesh

MATERIALS AND METHODS

Study population, sampling and samples

About 50% of village smallholdings in Bangladesh have bovine animals [21], although their numbers vary. They live close to human dwellings, exposing human drinking water and food to faecal contamination. We investigated SN-F STEC in cattle on such rural smallholdings in Bangladesh. One of the six divisions of Bangladesh, Chittagong, in the South-East, was selected for sampling. Eleven districts were used as the sampling frame from which three were selected by lottery: Chittagong, Feni and Noakhali. In hierarchical order these three districts were the first stratum of sampling; also sampled within these districts were eight sub-districts (*upazilas*) (second stratum), within these sub-districts 21 villages (primary sampling units), within these villages 371 cattle smallholdings, and finally within these smallholdings 518 individual animals. The proportion of cattle harbouring SN-F STEC was assessed at the overall population level, not by distinct clusters based on the differences in herd size because such differences are small as most cattle smallholdings in Bangladesh have only 1–3 animals.

From each smallholding at least one animal was sampled. For each animal a sterile swab was inserted into the anus and the rectal wall was swabbed. The swab was placed in a 5 ml tube containing buffered peptone water (Oxoid, UK), and sent to the Microbiology Laboratory, Chittagong Veterinary and Animal Sciences University (ML-CVASU), Bangladesh. Additional demographic and epidemiological information (age, sex, breed, health status and history of use of antibiotics) from each smallholding was collected.

Bacteriological investigations

For initial screening of SN-F STEC, CT-SMAC agar (Oxoid) was used, which is a selective medium for STEC O157 serogroup, indicated by the growth of colourless colonies [22–24]. Each sample was streaked onto a CT-SMAC agar plate, incubated at 37 °C for 24 h. At least five colourless colonies were transferred to a 10 ml test tube containing 5 ml of tryptic soy broth (TSB, Oxoid), and incubated at 37 °C for 6 h with continuous shaking. The growth in TSB was inoculated onto CT-SMAC agar again and when only homogeneous colourless colonies (Fig. 1) were observed on it, multiple cross-sectional colonies were verified for *E. coli* based on standard bacteriological procedures. Confirmed *E. coli* isolates were preserved at −80 °C in LB broth with 15% glycerin.

Verification of O157 serogroup and detection of shiga toxin-producing and other virulence genes

Each SN-F *E. coli* isolate was screened by polymerase chain reaction (PCR) for the *rfb* gene to verify

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Table 1. *Sequences of the primer sets used to detect five genes:* rfb, stx1, stx2, eae *and* hlyA *in sorbitol non-fermenting* Escherichia coli *from cattle on smallholdings in Bangladesh*

Primer	Primer sequence (5′–3′)	Target gene	Annealing temp. (°C)	Amplicon size (bp)	Ref.
rfb F	CGG ACA TCC ATG TGA TAT GG	*Rfb* O157	58	~259	[25]
rfb R	TTG CCT ATG TAC AGC TAA TCC				
stx1 F	ACA CTG GAT GAT CTC AGT GG	*stx1*	58	~614	[26]
Stx1 R	CTG AAT CCC CCT CCA TTA TG				
stx2 F	CCA TGA CAA CGG ACA GCA GTT	*stx2*	58	~779	[27]
stx2 R	CCTGTCAACTGAGCAGCACTT T				
eae F	CCCGAATTCGGCACAAGCATAAGC	*eae*	59	~881	[28]
eae R	CCCGGATCCGTCTCGCCAGTATTCG				
hlyA F	ACG ATG TGG TTT ATT CTG GA	*hlyA*	58	~165	[26]
hlyA R	CTT CAC GTG ACC ATA CAT AT				

whether it belonged to the O157 serogroup. The presence of two shiga toxin-producing genes, s*tx1* and s*tx2* and two other virulence genes, *eae* and *hly*A was also investigated by PCR. The sequences of five sets of primers used for the detection of these five genes and their amplicon sizes are shown in Table 1.

For PCR, DNA from the selected SN-F colonies was extracted by boiling, as described by Sánchez *et al.* [29]. Briefly, a loopful of bacterial growth was suspended in 0·5 ml deionized water, boiled for 5 min to release the DNA, and centrifuged at 15 000 ***g*** for 2 min, next 100 μl of the supernatant was collected in another tube to be used as DNA template. Uniplex PCR was performed separately for the detection of each of the five genes. Amplification of a gene was performed in 50 μl of reaction mixture containing 5 μl of 20 mM magnesium chloride, 1 μl of 40 μM dNTPs, 1 μl of 20 pmol of each primer, 0·2 μl of Dream *Taq* DNA polymerase (0·4 U/μl) (Thermo Scientific, Fermentas International Inc., USA), 1 μl of DNA template and 40·8 μl of molecular grade water. The conditions for PCR consisted of an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 40 s, and extension at 72 °C for 1 min, performed on a Thermo-cycler (2720 Thermal cycler, Applied Biosystems, USA). The amplicon from a PCR for a particular gene was analysed by electrophoresis on a 1·5% agarose gel stained with ethidium bromide (0·5 μg/ml) (Sigma-Aldrich, USA), and visualized by a UV trans-illuminator (BDA digital, Biometra GmbH, Germany). The amplicon size of a gene was determined by including a 1 kb size DNA marker (O'GeneRuler 1 kb plus; Thermo Scientific Fermentas, USA) in each gel. DNA from a previously isolated strain harbouring the particular gene, preserved at ML-CVASU was used as a positive control. A blank reagent having all components except the template DNA was used as a negative control.

Testing for clonal relationship of the isolates without virulence genes

Seven of the eight strains that had none of the virulence genes (*stx1, stx2, eae, hlyA*) investigated in this study were sent to the Department of Veterinary Disease Biology, Copenhagen University, Denmark, and investigated for clonal relationship by pulsed-field gel electrophoresis (PFGE) following the standardized CDC PulseNet protocol [30]. Briefly, an overnight culture of bacteria grown on brain heart infusion (BHI) broth (Oxoid) was used. Genomic DNA was prepared using 1% agarose (SeaKem® gold agarose, Lonza, USA) and embedded DNA was digested using 60 U of restriction enzyme *Xba*I (New England BioLabs Inc., USA) for 14 h at 37 °C. The DNA fragments were isolated by electrophoresis in 0·5x TBE buffer using the CHEF DR III (Bio-Rad Laboratories, USA) system at 14 °C with initial switch time 2·2 s, final switch time 54·2 s, current 6 V/cm, included angle 120° and run time 19 h. The gel was stained with 1% ethidium bromide (Sigma-Aldrich) solution for 30 min and destained in deionized water three times at 20-min intervals. Using UV transillumination, a gel image was captured by the GelDoc EQ system with Quantity One® v. 4.2.1 software (Bio-Rad Laboratories) and images were saved in TIFF format in a computer. The analysis of the fingerprints was performed using GelCompar® II v. 4.6 software (Applied Maths, Belgium). Dice coefficient with a band position

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Table 2. *Sampling levels – hierarchically from district to village, numbers of cattle sampled on the selected smallholdings and the corresponding number of sampled animals positive for shiga-toxin producing sorbitol non-fermenting* Escherichia coli *(SN-F STEC) in the study*

Level			No. smallholdings sampled	No. animals sampled	No. positive for SN-F STEC
District	Sub-district	Village			
1	1	1	11	24	4
1	2	2	15	27	7
1	3	3	14	27	4
1	4	4	13	21	0
1	1	5	10	20	0
1	1	6	9	20	0
1	4	7	12	20	1
2	5	8	15	20	0
2	6	9	20	23	0
2	6	10	22	26	0
2	6	11	18	21	0
2	7	12	25	29	0
2	7	13	17	22	0
2	7	14	19	30	2
3	8	15	16	27	1
3	8	16	24	31	1
3	8	17	21	26	3
3	8	18	27	35	2
3	8	19	18	20	1
3	8	20	26	26	1
3	8	21	19	23	1
Total: 3	8	21	371	518	28

District 1, Chittagong; district 2, Feni; district 3, Noakhali [there are 64 districts and 482 sub-districts (*upazilas*) in Bangladesh].

tolerance of 1% and 0·5% optimization level were used to determine similarity between fingerprints. The unweighted pair-group method with arithmetic averages (UPGMA) was applied to produce the dendrogram. The DNA restriction patterns of the isolates were interpreted as described by Tenover *et al.* [31].

Statistical analysis

All data were entered into a spreadsheet program (Excel 2003, Microsoft Corporation, USA) and transferred to Stata v. 11.0 (Stata Corp., USA) for analysis. The proportion of cattle positive with a particular phenotype/genotype of *E. coli* was calculated based on the number of animals found positive with this phenotype/genotype as the numerator and the total number of animals sampled as the denominator. The 95% confidence interval (CI) of a proportion estimate was calculated using the syntax 'cii #exposure #events, level (95) wilson'.

RESULTS

Overview of the study population

In a hierarchical order the sampling levels are shown in Table 2 with the villages as the primary sampling units. The numbers of bovine animals sampled on the selected smallholdings in the villages and the corresponding number of sampled animals positive for SN-F STEC are also presented. The number of smallholdings sampled was 371 and the total number of cattle owned by the selected households on the days of sampling was 789. The median number of cattle held was two (minimum one, maximum six). Of the 518 cattle sampled, 448 were local/indigenous (non-descriptive) type, and 70 cross-bred from indigenous dams.

Proportion of cattle positive with SN-F STEC

A total of 127 swabs yielded multiple SN-F (colourless) colonies on CT-SMAC agar plates, interspersed

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Table 3. *Distribution of shiga toxin producing genes,* stx1 *and* stx2*, and two other virulent genes,* eae *and* hlyA*, in the 44 sorbitol non-fermenting strains of* Escherichia coli *resulting from a survey encompassing 518 cattle on smallholdings in Bangladesh using cefixime potassium tellurite sorbitol MacConkey agar as the initial screening medium*

		Distributed by		PCR results for the targeted genes				
Isolate ID	District	Sex	Age	*stx1*	*stx2*	*hlyA*	*eae*	*rfb*
3*	1	M	A	–	+	+	+	–
4	1	M	A	–	–	–	+	–
11	1	F	A	–	–	+	+	–
13	1	F	C	–	+	–	–	–
15*	1	F	A	+	–	+	+	–
17	1	M	C	–	+	+	+	–
19	1	F	A	–	+	+	+	–
20*	1	F	A	–	+	+	+	+
21*	1	F	A	+	+	+	+	–
23*	1	M	C	–	+	+	+	+
27	1	M	A	–	+	–	–	–
29	1	M	C	–	+	–	+	–
30	1	F	A	–	–	+	+	–
31	1	M	A	–	+	–	–	–
34	1	F	C	–	–	+	–	–
35	1	M	C	–	+	–	–	–
40	1	M	A	–	+	–	+	–
42	1	M	C	–	+	–	+	–
44*	1	M	A	+	+	+	+	–
47	1	M	A	–	–	–	+	–
66*	1	F	A	–	+	–	–	–
77	1	F	A	–	–	–	+	–
82	2	F	A	–	+	–	+	–
83	2	M	C	–	–	–	+	–
84	2	F	A	–	+	–	–	–
85	3	M	C	–	+	+	–	–
87*	3	F	A	+	+	+	+	–
88	3	F	A	–	+	–	+	–
101	3	M	A	–	+	–	–	–
111	3	F	C	–	–	–	+	–
112*	3	F	A	+	–	+	+	–
121	3	M	A	–	+	–	+	–
129	3	M	A	–	+	–	+	–
134	3	M	A	–	+	+	–	–
135	3	F	A	–	+	–	+	–
155	3	F	A	–	+	+	–	–
1/Z 01	1	F	A	–	–	–	–	–
6/Z 02	1	M	A	–	–	–	–	–
10/Z 03	1	M	A	–	–	–	–	–
91/Z 04	2	F	C	–	–	–	–	–
94/Z 05	2	F	A	–	–	–	–	–
107/Z 06	3	F	A	–	–	–	–	–
114/Z 07	3	M	A	–	–	–	–	–
117/Z 08	3	F	C	–	–	–	–	–
Total				5	26	16	25	2

A, Adult; C, calf; +, presence of the gene; –, absence of the gene; * isolates: amplicons of the respective investigated genes which are displayed in Figure 2.
District 1, Chittagong; district 2, Feni; district 3, Noakhali.

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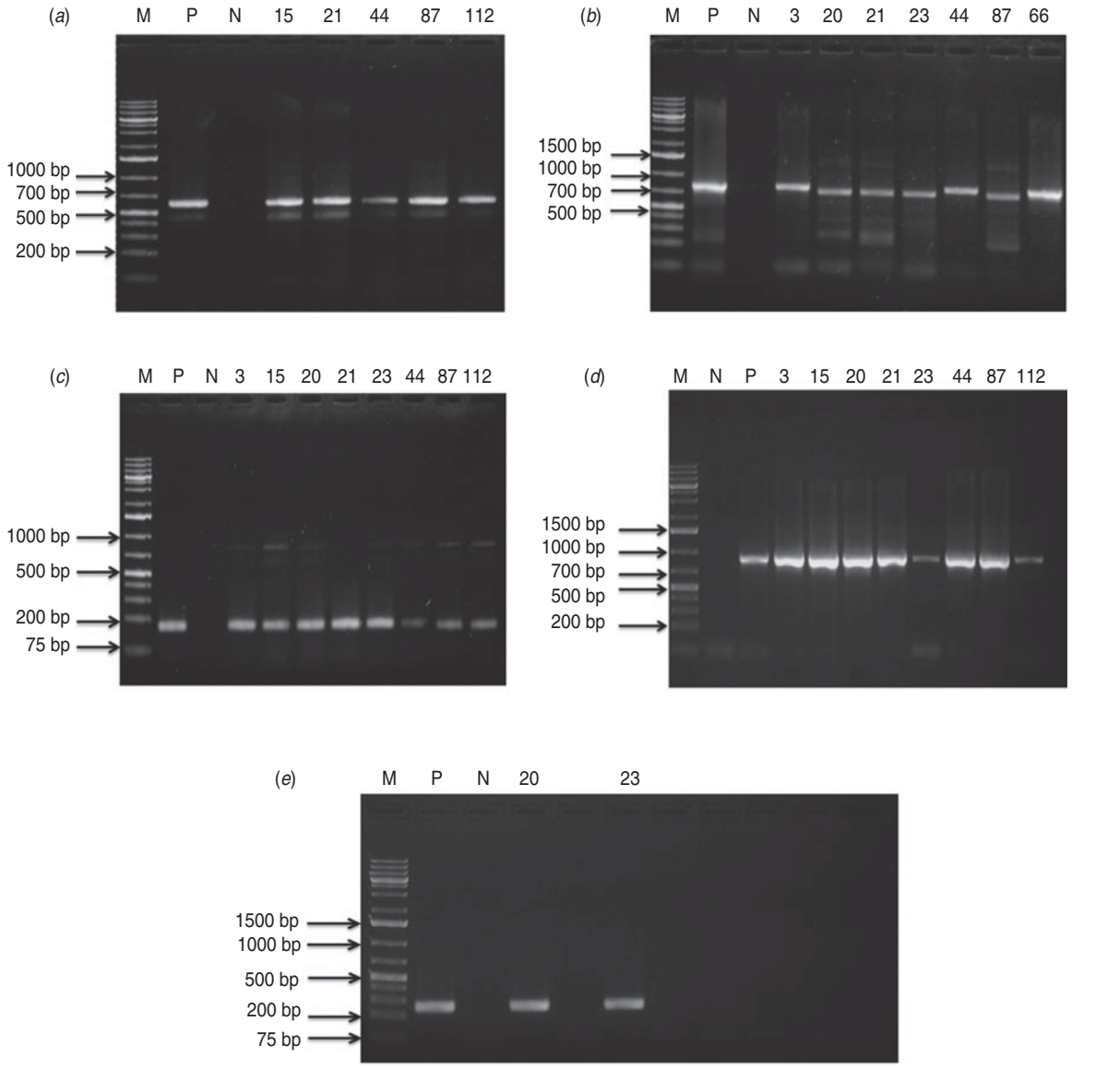

Fig. 2. Results of polymerase chain reaction assays displaying amplicons of the shiga toxin 1-producing gene (*stx1*) in five (*a*), shiga toxin 2-producing gene (*stx2*) in seven (*b*), enterohaemolysin-producing gene (*hlyA*) in eight (*c*), intimin-producing gene (*eae*) in eight (*d*) and a somatic antigen-producing gene indicating presence of any member belonging to O157 serogroup (*rfb*) in two (*e*) of the sorbitol non-fermenting *Escherichia coli* strains (indicated by an asterisk in Table 3) from cattle on smallholdings in Bangladesh. Lane M, 1 kb plus DNA marker; lane P, positive control isolates having the specific gene of the concerned panel; lane N, negative control.

with others including sorbitol-fermenting (SF) (pink) ones. When cultures from at least five colourless homogeneous colonies in TSB were plated onto CT-SMAC agar, 57 yielded pure SN-F (colourless) colonies, and 44 were identified as *E. coli*, giving a proportion of 8·5% (95% CI 6·4–11·2). Only two isolates were positive for the *rfb* gene, a very low proportion (0·4%, 95% CI 0·1–1·4) of the O157 serogroup; however, five and 26 isolates carried the *stx1* and *stx2* genes, respectively (Table 3); hence, the proportion of cattle carrying SN-F shiga toxin 1- and shiga toxin 2-producing *E. coli* was 0·9% (95% CI 0·4–2·2) and 5% (95% CI 3·4–7·3), respectively. Of the STEC isolates three had both *stx1* and *stx2* genes; therefore, a total of 28 samples (5·4%, 95% CI 3·8–7·7) were positive for STEC. Of the 28 STEC, irrespective of whether they harboured *stx1* or *stx2* or both genes, 13 were also positive for the *hlyA* gene, and 18 carried the *eae* gene. On the other hand, eight SN-F non-STEC isolates were positive for the *hlyA* and/or the *eae* genes. PCR results

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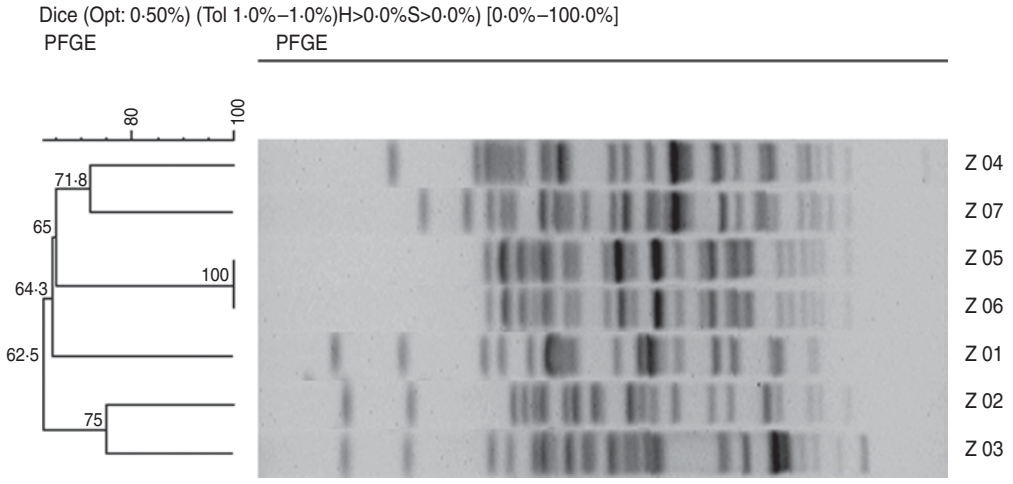

Fig. 3. Dendrogram showing the cluster analysis on the basis of *Xba*I pulsed-field gel electrophoresis (PFGE) of the seven sorbitol non-fermenting *Escherichia coli* isolates from cattle on smallholdings in Bangladesh lacking the shiga toxin genes, *stx1* and *stx2* and two other virulence genes *eae* and *hlyA*. Dice coefficient was used to perform similarity analysis, and clustering was performed by using The unweighted pair-group method with arithmetic means with 1% band position tolerance and 0·5% optimization parameter.

illustrating amplicons of some SN-F *E. coli* isolates harbouring *stx1*, *stx2*, *hlyA*, *eae* and *rfb* genes are shown in Figure 2(*a–e*).

According to geography 16, two and 10 animals were found positive for STEC in districts 1 (Chittagong), 2 (Feni) and 3 (Noakhali), respectively. In these animals, the male:female ratio was 15:13, with 21 adults and seven calves.

Clonal relationship of the isolates lacking any shiga toxin, *hlyA* and *eae* genes

Among the seven isolates tested, six different pulsotypes were identified based on the variations in >9 bands (i.e. >3 genes) (Fig. 3).

DISCUSSION

Approximately 5·4% of cattle on smallholdings in the study area carry SN-F STEC. Of the 28 SN-F STEC isolates 13 (46·4%) and 18 (64·3%) had the virulence gene *hlyA* and *eae*, respectively. The proportion harbouring STEC O157 appears to be negligible, only 0·4%. Based on the distributions of two shiga toxin-producing genes *stx1* and *stx2*, and the presence of the two virulence genes *eae* and *hlyA*, the SN-F STEC isolates obtained appear highly diverse. The SN-F *E. coli* isolates lacking any of the four virulence genes – *stx1*, *stx2*, *eae* and *hlyA* – were genetically diverse. To the authors' knowledge, this might be the first report on SN-F STEC from cattle on smallholdings from Bangladesh or any part of the developing world, to analyse faecal samples collected directly from the rectum.

The aim of the study was to assess the proportion of cattle harbouring STEC at the overall population level, not its carriage rate at any cluster or subpopulation level, on the basis of herd size or different exposure variables such as age, breed, sex, health status and use of antibiotics. A higher sample size from a specific sub-population is required to estimate the prevalence of STEC in that population.

By the use of CT-SMAC we sought to isolate all bacteria belonging to the STEC O157 serogroup (a widely reported serogroup that can cause colitis, HUS and sometimes death in humans) [4, 5] and other SN-F types. This study demonstrates that based on seeing colourless colonies on CT-SMAC, STEC O157 cannot be reliably differentiated from other organisms; therefore other tests must be applied. We used PCR to detect the *rfb* gene, responsible for the production of a common somatic antigen of the O157 serogroup, and only two of the 44 SN-F isolates obtained from the survey belonged to the STEC O157 serogroup. However, both the isolates harboured only one shiga toxin-producing gene, i.e. *stx2*.

To increase the probability of isolating STEC O157 or any members of STEC belonging to other serogroups, we examined multiple ($\geqslant 5$) SN-F colonies that were mixed with other colony types on CT-SMAC. However, SN-F *E. coli* was identified from only 44 of the 127 samples. Except for two

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isolates harbouring the *rfb* gene, O-serotypes of the other isolates were not determined because of resource constraints. Neither were the identities of SN-F bacterial isolates other than *E. coli* investigated, as this was considered beyond the scope of the study. However, among others *Burkholderia, Pseudomonas, Vibrio* and *Aeromonas* have been reported to produce SN-F colonies on CT-SMAC [32]. We did not investigate any of the 13 SN-F strains found to be repeatedly S-NF but non-*E. coli* for the presence of *hlyA* and/or *eae* genes. Despite the above-mentioned limitations of this study, an important finding of this investigation is that, on smallholdings in Bangladesh, the proportion of cattle harbouring SN-F non-O157 STEC might be much higher than the proportion positive with STEC belonging to serogroup O157.

Compared to shiga toxin 1-producing SN-F STEC isolates the proportion of shiga toxin 2-producing isolates was ~4 times higher in the study population (Table 3), in agreement with Tahamtan *et al.* [33] and El-Jakee *et al.* [34] who reported prevalence in cattle of 10% *vs.* 53% and 39% *vs.* 77%, respectively. The prevalence of the *stx2* gene in STEC in recently slaughtered cattle sampled at slaughterhouses in Bangladesh was reported to be >93%, although its presence, compared to the *stx1* gene in non-O157 STEC, was found to be lower [35]. SF STEC strains belonging to serotype O157:H$^-$ (non-motile) have emerged as important causes of human disease [36–38] with some evidence that they are more frequently associated with HUS than are SN-F strains [38, 39]. Because the aim of the study was to investigate shiga toxin-producing SN-F STEC, the rate at which the study population harboured SF STEC was not determined.

Because of unrepresentative sample sizes at the subpopulation level the isolation trends of STEC in adult animals compared to the calves and in males compared to females could not be predicted from this study. However, one male and two female animals belonging to three different smallholdings were found to be reservoirs for SN-F STEC carrying the *stx1, stx2* and *eae* genes, a potentially highly virulent combination [20].

Most STEC isolates resulting from this study were genetically diverse because of having different combinations of *hlyA, eae* and shiga toxin-producing genes (Table 2), as previously mentioned. Therefore, we did not apply PFGE to discriminate them further. However, Islam *et al.* [35] reported at least 37 different PFGE patterns in the STEC strains isolated from cattle slaughtered in Bangladesh, indicating heterogeneous clonal diversity. Because eight of the SN-F *E. coli* isolates lacking any virulence genes could not be discriminated by PCR we applied PFGE to verify whether or not any specific clone of such SN-F non-STEC was circulating in the study population, although the potential of SN-F non-STEC in causing human infections has probably not been documented. The PFGE results reveal that the investigated isolates were genetically diverse (Fig. 3).

In conclusion, ~8% and ~6% of cattle on smallholdings are reservoirs of SN-F *E. coli* and SN-F STEC, respectively. Most SN-F STEC are non-O157 and only ~0·4% might be STEC O157. Among SN-F STEC, 10·7% should be considered highly virulent to humans because of the *stx1, stx2* and *eae* genes, indicating a threat from cattle on smallholdings to the people sharing the same homestead. The proportion of cattle positive with SF STEC remains unknown. More studies are needed to assess the prevalence of SF STEC and to identify the sources and risk factors associated with SN-F or SF STEC from cattle on smallholdings in human infection.

ACKNOWLEDGEMENTS

The authors thank W. R. Ward for examining the manuscript. The study was financed by RFLDC, Noakhali though the Poultry Research and Training Centre, CVASU, Bangladesh. Thanks are extended to Dr Himel Barua for providing valuable technical support.

DECLARATION OF INTEREST

None.

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