Prevalence of sorbitol non-fermenting Shiga toxin-producing *Escherichia coli* in Black Bengal goats on smallholdings

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SUMMARY

A cross-sectional survey was carried out in Bangladesh with the sampling of 514 Black Bengal goats on smallholdings to determine the presence of sorbitol non-fermenting (SNF) Shiga toxin-producing *E. coli* (STEC). Swab samples collected from the recto-anal junction were plated onto cefixime and potassium tellurite added sorbitol MacConkey (CT-SMAC) agar, a selective medium for STEC O157 serogroup, where this serogroup and other SNF STEC produce colourless colonies. The SNF *E. coli* (SNF EC) isolates obtained from the survey were investigated by PCR for the presence of Shiga toxin-producing genes, *stx1* and *stx2*, and two other virulence genes, *eae* and *hlyA* that code for adherence factor (intimin protein) and pore-forming cytolysin, respectively. The SNF EC isolates were also assessed for the presence of the *rfbO157* gene to verify their identity to O157 serogroup. The results revealed that the proportions of goats carrying SNF EC isolates and *stx1* and *stx2* genes were 6·2% (32/514) [95% confidence interval (CI) 4·4–8·7)], 1·2% (95% CI 0·5–2·6) and 1·2% (95% CI 0·5–2·6), respectively. All the SNF STEC tested negative for *rfbO157, hlyA* and *eae* genes. The risk for transmission of STEC from Black Bengal goats to humans is low.

Key words: Black Bengal goats, *E coli*, shiga toxin.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are important human pathogens. STEC including serotype O157:H7 and other non-O157 serogroups cause serious illness, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS), as well as death in humans [1]. The pathogenicity of STEC is chiefly mediated through the Shiga toxins (*stx1* or *stx2* or their variants) encoded by the *stx1* and *stx2* genes [2]. Besides the *stx* gene(s), human pathogenic

STEC strains often carry the *eae* gene, encoding the adherence factor, i.e. the 'intimin' protein, which is involved in the intimate adhesion of bacteria to enterocytes, with the production of attaching and effacing lesions [3]. STEC strains may also harbour a large plasmid encoding additional virulence factors, such as the haemolysin (*hly*) gene, which acts as a pore-forming cytolysin on eukaryotic cells [4].

Although healthy cattle are the widely studied natural reservoir for STEC, other domestic ruminants, mainly sheep and goats, have also been reported as asymptomatic carriers for STEC, and thus they might play an important role in human infections [5]. STEC O157:H7 is the most prevalent serotype causing human infections worldwide, and based on

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sorbitol-non fermentation property its presence in fae-cal samples could be primarily screened on a select-ive medium sorbitol MacConkey agar supplemented with cefixime and potassium tellurite (CT-SMAC). Recently, sorbitol non-fermenting (SNF) STEC was, for the first time, reported in cattle on smallholdings in Bangladesh [6]. However, Black Bengal goats, reared on smallholdings, particularly in Bangladesh and India have seemingly not been studied for the presence of this important zoonotic pathogen.

Goats represent the fourth largest livestock group in the world [7]. Of about 860 million goats reared world-wide 18 million are in Bangladesh [7] and >90% of them are of the Black Bengal breed [8], reared mainly by land-less, small-scale farmers primarily for meat [9]. The mean number of goats per smallholding in Bangladesh is 2·3 [9] and this small ruminant plays an important role in the rural economy of Bangladesh and parts of India. This breed is considerably disease-tolerant, but its role in har-bouring STEC and its dispersion to human food and water sources have never been studied.

SNF STEC displays delayed (negative) fermenta-tion of D-sorbitol, and is therefore commonly isolated by growing on CT-SMAC [10, 11]. In addition, poly-merase chain reaction (PCR) assay provides a satisfac-tory sensitivity for identification of virulence gene(s) from SNF isolates [12]. Here, we describe the propor-tions of Black Bengal goats in smallholdings carrying SNF *Escherichia coli* (SNF EC), SNF STEC and the presence of two other virulence genes, *eae* and *hlyA* in the SNF EC strains isolated from them.

MATERIALS AND METHODS

Black Bengal goats

The Black Bengal goat is a dwarf-type, highly prolific breed of goat [13]. Although the predominant coat colour is black, brown, grey and white are also seen. Castrated males aged 1 year and females of the same age weigh about 16 kg and 12 kg, respectively. Both sexes have cylindrical horns, and beards at older ages. Sexual maturity is attained at 6–8 months, and they reproduce twice a year even with a low level of nutrition: the average litter size is 2·2 [13]. These animals are considerably disease tolerant [13].

Study population and sample collection

We performed a cross-sectional prevalence study using multi-stage sampling. For sampling, we selected one of the six divisions of Bangladesh – Chittagong. The hierarchical stratum for sampling included two districts – Chittagong and Noakhali; within these dis-tricts five sub-districts (locally known as *upazilas*) were chosen; within these sub-districts were 13 primary sampling points (villages). From these primary sam-pling points 514 Black Bengal goats were sampled from June 2011 to June 2012. Because the aim of the study was to examine the proportion of Black Bengal goats in smallholdings carrying SNF STEC, and because the rearing system and management of Black Bengal goats in smallholdings is the same across the country, the sampling division mentioned above was conveniently selected. However, the primary sam-pling unit(s) [village(s) under a sub-district]] were selected based on the information provided from the corresponding Upazila Livestock Office on village(s) where a significant section of villagers reared Black Bengal goats. In order to increase the representative-ness of the sample we sampled 13 villages located in five different sub-districts.

To the authors' knowledge there is no published re-port on the proportion of live Black Bengal goats positive for STEC. However, Islam *et al.* [14] reported that its proportion in slaughtered goats, irrespective of breed, could be 10%. With this proportion, the min-imum number of goats required for sampling was 258 with 90% power at the 5% level of significance [15]. However, we sampled 514 goats, with at least 25 from each sampling point.

The samples were collected from the recto-anal junction using sterile swabs which were placed in a 5 ml tube containing buffered peptone water (Oxoid, UK). The samples were transported immediately to the Microbiology Laboratory, Chittagong Veterinary and Animal Sciences University, Bangladesh. In add-ition, we collected demographic information on the animals sampled.

Culture and STEC isolation

A SNF EC isolate was primarily detected based on their sorbitol non-fermentation (colourless colonies) using a selective medium (CT-SMAC agar; Oxoid). Each sample was inoculated on CT-SMAC agar and incubated at 37 °C for 24 h. For enrichment, at least five colourless colonies from CT-SMAC agar were inoculated onto tryptic soy broth (TSB), incubated at 37 °C for 6 h in a shaker incubator. Growths from TSB were subcultured on CT-SMAC agar again and five cross-sectional colonies from

Table 1. *Oligonucleotide primers used in PCR for the detection of five virulent genes* rfbO157, stx1, stx2, eae *and* hlyA

Primer	Primer sequence (5′–3′)	Target gene	Annealing temp. (°C)	Size of product (bp)	Reference
rfb F	CGG ACA TCC ATG TGATAT GG	*rfbO157*	58	~259	Desmarchelier *et al.* 1998 [16]
rfb R	TTG CCT ATG TAC AGC TAA TCC				
stx1 F	ACA CTG GAT GAT CTC AGT GG	*stx1*	58	~614	desRosiers *et al.* 2001 [17]
stx1 R	CTG AAT CCC CCT CCA TTA TG				
stx2 F	CCA TGA CAA CGG ACA GCA GTT	*stx2*	58	~779	Manna *et al.* 2006 [18]
stx2 R	CCT GTC AAC TGA GCA GCA CTT T				
eae F	CCCGAATTCGGCACAAGCATAAGC	*eae*	59	~881	Oswald *et al.* 2000 [19]
eae R	CCCGGATCCGTCTCGCCAGTATTCG				
hlyAF	ACG ATG TGG TTT ATT CTG GA	*hlyA*	58	~165	desRosiers *et al.* 2001 [17]
hlyAR	CTT CAC GTG ACC ATA CAT AT				

homogenous colourless growth in secondary culture [6] were inoculated onto Eosin Methylene Blue (EMB, Oxoid) agar plates to look for the characteristic metallic green sheen produced by *E. coli*. Isolates with a metallic green sheen were confirmed as *E. coli* and preserved at −80 °C in LB broth with 15% glycerin until assessment for virulence genes. A representative number of these isolates were further tested for the presence of seven housekeeping genes of *E. coli*: *adk, mdh, purA, gyrb, icd, recA* and *fumC*, by PCR using the recommended primers and the protocol available at http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html.

Detection of Shiga toxin-producing genes (*stx1 and stx2*) and two other virulence genes (*eae* and *hlyA*)

All the SNF EC isolates were first assessed for the presence of the *rfbO157* gene to verify whether they belonged to the O157 serogroup, and then for *stx1, stx2, eae* and *hlyA* genes, by PCR. Oligonucleotide primers used to detect these five genes and their predicted product sizes are given in Table 1. Each SNF EC isolate was grown in 5% citrated bovine blood agar, and DNA was extracted using a boiling method described by Sánchez *et al.* [20]. Briefly, one loopful of colonies of each isolate was suspended in 0·5 ml deionized water, boiled for 5 min to release DNA, and the boiled suspension was centrifuged. The supernatant was used as DNA template in PCR reactions. Uniplex PCR was used for detection of each virulence gene. Amplification of bacterial DNA was performed using 50 μl volumes containing 1 μl of the prepared sample supernatant; 1 μl (each) oligonucleotide primer (20 pmol), 5 μl of 20 mM magnesium chloride, 1 μl of

40 μM dNTPs, 0·2 μl DreamTaq DNA polymerase (0·4 U/μl) (Thermo Scientific, Fermentas International Inc., USA), 1 μl DNA template and 40·8 μl molecular grade water. The conditions for the PCR were 95 °C for 3 min for initial denaturation of DNA followed by 35 cycles at 95 °C for 20s (denaturation), 58–59 °C for 40s (primer annealing) and 72 °C for 1 min (DNA synthesis) performed on a Thermo-cycler (2720 Thermal cycler, Applied Biosystems, USA). The amplified product for a specific gene was electrophoresed using 10 μl of the final reaction mixture on a 1·5% agarose gel stained with ethidium bromide (0·5 μg/ml) (Sigma-Aldrich, USA). The DNA fragment of specific sizes was located by a UV transilluminator (BDA digital, Biometra GmbH, Germany). A molecular size marker (O'GeneRuler 1 kb Plus, Thermo Scientific Fermentas) was included in each gel. A SNF STEC strain isolated by Islam *et al.* [6] was used as a positive control.

Statistical analysis

The proportion of Black Bengal goats positive for SNF EC or SNF STEC was calculated based on the number of animals found positive with the corresponding type (numerator), divided by the total number of animals sampled (denominator) using Graph Pad software quick calculation for 'Confidence interval of a proportion by modified Wald method' (http://www.graphpad.com/quickcalcs/confInterval2/). The 95% confidence interval (CI) of a proportion and significance in the difference of the proportions for SNF EC or SNF STEC in two age groups (adults and kids) and sexes, were assessed by Fisher's exact test using Graph Pad software [21].

Table 2. *Sampling strata (hierarchically from district to sampling unit), the corresponding number of sampled Black Bengal goats and animals carrying Shiga toxin-producing sorbitol non-fermenting* Escherichia coli *(SNF STEC) in the study*

Stratum				
District	Sub-district	Sampling unit	No. animals sampled	No. animals carrying SNF STEC
1	1	1	94	2
1	2	2	25	1
1	3	3	31	2
1	3	4	25	1
1	3	5	25	1
1	4	6	29	1
2	5	7	58	2
2	5	8	50	1
2	5	9	48	1
2	5	10	29	0
2	5	11	44	0
2	5	12	25	0
2	5	13	31	0
Total: 2	5	13	514	12

District 1, Chittagong; district 2, Noakhali. (There are 64 districts and 482 sub-districts in Bangladesh.)

RESULTS

Summary of the study population

The summary of the sampling strata in hierarchical order from district to sampling point and the corresponding number of animals sampled and animals carrying SNF STEC are given in Table 2. The number of animals sampled from a sampling point varied from 25 to 94. Of the 514 goats sampled, 367 were adults (>6 months) while 147 were kids (<6 months); 212 were male and 302 were female: 214 goats were from Chittagong (district 1) and 300 from Noakhali (district 2).

Proportion of goats carrying SNF EC and SNF STEC

Of the 514 swab samples plated onto CT-SMAC agar, 79 yielded multiple SNF (colourless) colonies interspersed with sorbitol fermenting (SF) pink colonies. Secondary growth by plating cultures through TSB onto CT-SMAC agar yielded 41 pure SNF isolates, of which 32 were finally identified as *E. coli*.

The results of uniplex PCR showed that no isolate was positive for the *rfb* gene; however, six were positive for the *stx1* gene and six for the *stx2* gene.

Therefore, the proportion of animals harbouring SNF EC containing *stx1* or *stx2* genes (i.e. SNF STEC) was 1·2% (6/514) (95% CI 0·5–2·6) and 1·2 % (6/514) (95% CI 0·5–2·6), respectively. None of the isolates carried both genes, and thus, in total, 12 (2·3%, 95% CI 1·3–4·1) goats were positive for SNF STEC. All the 12 SNF STEC isolates were negative for the two other virulence genes (*hlyA* and *eae*) tested; however, seven and two isolates (not harbouring *stx1* or *stx2* genes) were positive for *hlyA* and *eae* genes, respectively. PCR results displaying the gene-specific amplicons of some SNF STEC and SNF EC isolates are depicted in Figure 1(*a–d*).

Because the test sensitivity and specificity were not accounted for in the prevalence calculation, the prevalence estimates for animals carrying SNF STEC either with the *stx1* or *stx2* genes were their apparent prevalence estimates (proportion of animals that were positive by the diagnostic method), and not the true prevalence estimates. In district 1 (Chittagong) and district 2 (Noakhali) eight and four animals, respectively, tested positive for SNF STEC. Ten adult goats and two kids (<6 months) were positive with SNF STEC ($P = 0·352$); five were male and seven female ($P = 0·976$) (Table 3).

DISCUSSION

In this study, we report the presence of SNF EC including STEC in Black Bengal goats in Bangladesh. Approximately 6% (4·4–8·7%) and 2% (1·3–4·1%) of the Black Bengal goats in the study area were positive for SNF EC and SNF STEC, respectively, indicating a low proportion of this breed harbouring SNF STEC. Estimation of the confidence intervals did not account for the sampling design (clustering of sampled animals in villages), and, therefore, that confidence intervals are likely to be underestimated. In a previous study, Islam *et al.* [6] reported that 8·5% and 5·4% of cattle on smallholdings were positive for SNF EC and SNF STEC, respectively, thus suggesting that the probability of isolation of SNF STEC from Black Bengal goats is nearly half of that from cattle. This lower presence of STEC in goats compared to cattle is corroborated by other studies [14, 22]. However, a higher prevalence (10%) of STEC in slaughtered goats, irrespective of breed, was reported by Islam *et al.* [14]. This difference might be linked to differences in the status (dead or alive) of animals sampled, breed and age of goats [23, 24], the procedures used to isolate the organisms,
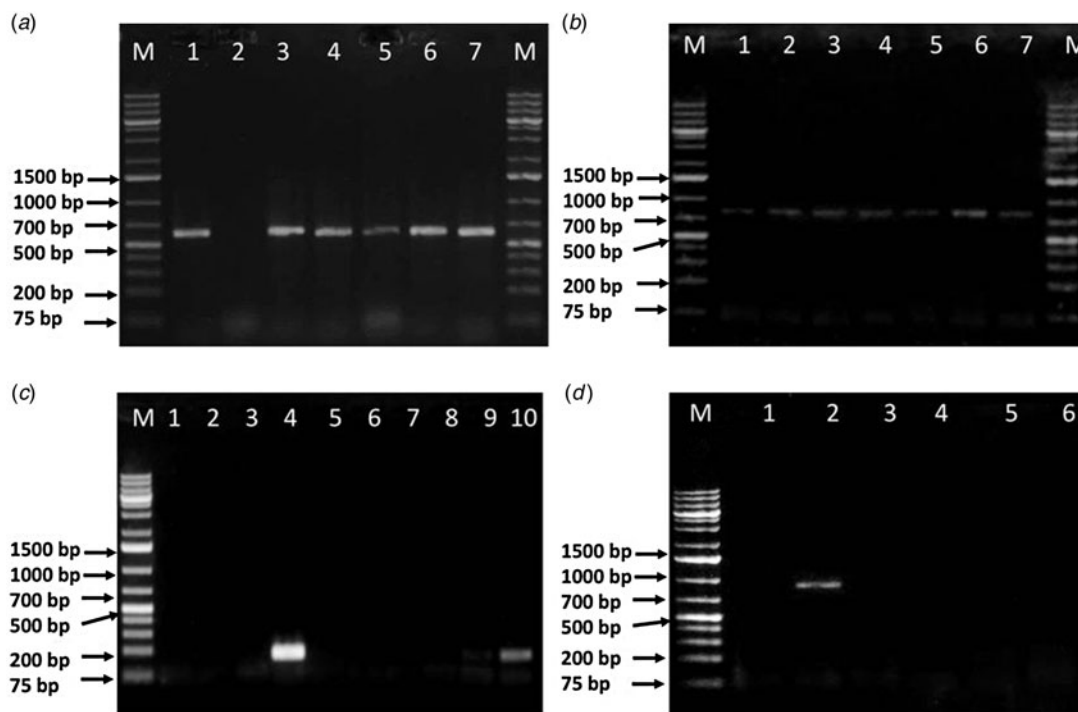
Fig. 1. Results of PCR assay for four virulent genes: *stx1, stx2, hlyA* and *eae*. (*a*) *stx1* gene (614 bp) amplicon: lane M = 1 kb plus DNA ladder; lanes 1, 3–7, *stx1*-positive isolates; (*b*) *stx2* gene (779 bp) amplicon: lane M = 1 kb plus DNA ladder; lanes 1–7, *stx2*-positive isolates; (*c*) *hlyA* gene (165 bp) amplicon: lane M = 1 kb plus DNA ladder; lanes 4, 9–10, *hlyA*-positive isolates; (*d*) *eae* gene (881 bp) amplicon: lane M = 1 kb plus DNA ladder; lane 2, *eae*-positive isolate.

and spread of organism between animals in abattoir. The proportion of Black Bengal goats in the present study that harboured STEC was lower than reported from goats of other breeds in Southern Jordan (6·4%) [25], England & Wales (6·1%) [26], Malaysia (8·14%) [27], Spain (16·2%) [28] and India (16·7%) [29]. These differences probably indicate that the carriage proportion of SNF STEC in Black Bengal goats is substantially lower than in other breeds.

The primary isolation based on the production of colourless colonies on CT-SMAC agar revealed that not all the 41 SNF isolates were *E. coli* or STEC; in fact, 32 of them were found to be *E. coli*. We did not perform any additional screening to identify the nine SNF isolates that were not *E. coli*. Simultaneously, the results revealed that identification of SNF STEC based on just observing colourless colonies on CT-SMAC should not be relied upon for goat faecal samples. Additional testing, such as PCR, to detect virulent genes or O-serotyping is required.

Using PCR, all the SNF isolates were negative for the *rfb* gene, suggesting that they did not belong to the STEC O157 serogroup, a commonly reported serogroup of STEC that causes severe acute human infection. A similar result, i.e. STEC of non-O157 serogroup

has been reported by other studies [22, 30, 31]. However, in a previous study [6], a very low proportion (0·4%) of cattle on smallholdings in Bangladesh was found positive for STEC O157 serogroup. The results of the present study indicate that Black Bengal goats probably do not carry SNF STEC belonging to O157 serogroup or, if carried, the proportion might be very low.

Six of the SNF STEC isolates, i.e. 1·2% of the goat isolates had the *stx1* gene alone, in agreement with Roy & Roberts [32] and Islam *et al.* [14]. The proportion of isolates that had the *stx2* gene was the same, 1·2%, in agreement with Wani *et al.* [29], who found that 2·9% of goats in India carried this virulence gene. However, contrasting results for the possession of *stx1* or *stx2* genes have been reported in previous studies [28, 33]. These variations in the prevalence of STEC possessing only one kind of Shiga toxin-producing gene might be influenced by different factors including diet, age, environmental conditions and seasonal variations [34].

The proportion of *E. coli* containing the *hlyA* gene was 1·4%, which is similar to that reported in cattle in smallholdings [6], whereas in goats, Islam *et al.* [14] and Wani *et al.* [29] reported higher proportions, 7%

Table 3. *Distribution of Shiga toxin-producing genes,* stx1 *and* stx2, *and two other virulent genes,* eae *and* hlyA, *in the 32 sorbitol non-fermenting strains of* Escherichia coli *isolated from Black Bengal goats in Bangladesh*

Isolate ID no.	District	Distributed by		PCR results for targeted genes				
		Age	Sex	*stx1*	*stx2*	*hlyA*	*eae*	*rfb*
9	2	K	M	−	−	+	−	−
33	2	A	M	−	−	−	+	−
48	2	A	M	−	+	−	−	−
71	2	A	F	−	+	−	−	−
85	2	A	F	+	−	−	−	−
96	2	A	F	−	−	−	−	−
103	2	A	F	−	−	−	+	−
119	2	A	F	−	−	−	−	−
142	2	A	M	−	−	+	−	−
192	2	A	F	+	−	−	−	−
202	2	A	F	−	−	+	−	−
229	2	A	M	−	−	+	−	−
249	2	A	F	−	−	+	−	−
267	2	A	F	−	−	+	−	−
294	2	K	F	−	−	+	−	−
299	2	A	F	−	−	−	−	−
328	1	K	M	−	−	−	−	−
336	1	A	F	−	−	−	−	−
341	1	A	M	−	−	−	−	−
370	1	K	F	+	−	−	−	−
379	1	K	M	−	+	−	−	−
384	1	A	F	−	−	−	−	−
405	1	A	F	+	−	−	−	−
415	1	A	M	+	−	−	−	−
427	1	A	M	−	−	−	−	−
438	1	A	F	−	+	−	−	−
449	1	A	F	−	−	−	−	−
472	1	A	F	−	−	−	−	−
478	1	A	F	−	+	−	−	−
492	1	A	M	−	+	−	−	−
505	1	A	M	+	−	−	−	−
510	1	K	M	−	−	−	−	−
			Total	6	6	7	2	

A, Adult; K, kid; +, presence of the gene; −, absence of the gene.
District 1, Chittagong; district 2, Noakhali.

and 14·7%, in Bangladesh and India, respectively. Only about 0·4% of goats were positive with SNF EC containing the *eae* gene, a similar finding to that reported by other studies [14, 23, 29, 35]. Orden *et al.* [24] reported VTEC isolates without the *eae* gene from healthy goats, except three *E. coli* O157 H7 isolates. Taken together, these reports and the results of the present study suggest that very few STEC from Black Bengal goats harbour the *eae* gene. Louie *et al.* [36] and Barrett *et al.* [37] reported that the *eae* gene is essential for full virulence expression of STEC in humans. However, Paton *et al.* [38] reported that the *eae* gene might be required to produce HC and HUS for a few strains of STEC, but not for others. Therefore, the *eae* gene-negative STEC of goat origin might also have the potential to cause human infection.

The distributions of goats carrying SNF STEC between the two sampled districts did not differ significantly. Similar husbandry for goats in smallholdings in the two districts might be the reason for this.

In terms of pathogenicity of STEC, the most virulent combination is the possession of both Shiga toxin-producing genes, *stx1* and *stx2*, and the *eae* gene in the same strain [39, 40], which was not detected in the Black Bengal goats sampled. However, any *E. coli* having a single Shiga toxin-producing gene has the potential to cause human infection [41–43]. Therefore, SNF STEC being harboured in Black Bengal goats, as observed in the present study, might have zoonotic transferability through contaminated food and water, although such an occurrence would seemingly be low compared to cattle in Bangladesh, as observed by Islam *et al.* [6].

CONCLUSION

The carriage proportion of SNF EC and SNF STEC in faeces of Black Bengal goats was about 6% and 2%, respectively. SNF EC or SNF STEC can be seen evenly distributed in adult goats and kids, irrespective of sex and geographical location. The presence of STEC strains having both *stx1* and *stx2* genes and the *eae* gene might be rare in the Black Bengal goat population. In Bangladesh, the public health threat from STEC in Black Bengal goats is seemingly low compared to that from cattle.

DECLARATION OF INTEREST

None.

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