Shiga Toxin-Producing *Escherichia coli* in Yaks (*Bos grunniens*) from the Qinghai-Tibetan Plateau, China

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Abstract

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are recognized as important human pathogens of public health concern. Many animals are the sources of STEC. In this study we determined the occurrence and characteristics of the STEC in yaks (*Bos grunniens*) from the Qinghai-Tibetan plateau, China. A total of 728 yak fecal samples was collected from June to August, 2012 and was screened for the presence of the stx_1 and stx_2 genes by TaqMan real-time PCR after the sample was enriched in modified Tryptone Soya Broth. Of the 138 (18.96%) stx_1 and/or stx_2-positive samples, 85 (61.59%) were confirmed to have at least 1 STEC isolate present by culture isolation, from which 128 STEC isolates were recovered. All STEC isolates were serotyped, genotyped by pulsed-field gel electrophoresis (PFGE) and characterized for the presence of 16 known virulence factors. Fifteen different O serogroups and 36 different O:H serotypes were identified in the 128 STEC isolates with 21 and 4 untypable for the O and H antigens respectively. One stx_1 subtype (stx_{1a}) and 5 stx_2 subtypes (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d} and stx_{2g}) were present in these STEC isolates. Apart from $lpfA_{O157/OI-141}$, $lpfA_{O157/OI-154}$, $lpfA_{O113}$, $katP$ and $toxB$ which were all absent, other virulence factors screened ($eaeA$, iha, $efa1$, saa, paa, $cnf1$, $cnf2$, $astA$, $subA$, $exhA$ and $espP$) were variably present in the 128 STEC isolates. PFGE were successful for all except 5 isolates and separated them into 67 different PFGE patterns. For the 18 serotypes with 2 or more isolates, isolates of the same serotypes had the same or closely related PFGE patterns, demonstrating clonality of these serotypes. This study was the first report on occurrence and characteristics of STEC isolated from yaks (*Bos grunniens*) from the Qinghai-Tibetan plateau, China, and extended the genetic diversity and reservoir host range of STEC.

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are recognized globally as major food-borne pathogens. Clinical manifestations of STEC infections in humans range from non-bloody diarrhea to hemorrhagic colitis (HC) and sometimes fatal hemolytic uremic syndrome (HUS) complications. There are more than 200 known STEC serotypes associated with human illness [1]. O157:H7 is the most frequently encountered STEC in human infections [2]. Many outbreaks and sporadic infections caused by STEC O157:H7/NM have been reported in different regions of the world [3,4,5,6,7,8]. However, non-O157 STEC isolates have been increasingly associated with human infections and outbreaks. In 2011, Germany experienced the largest outbreak of non-O157 STEC, O104:H4, ever recorded with 3,816 cases including 845 HUS cases and 54 deaths, similar outbreaks were reported in France and other counties in Europe subsequently [9,10,11,12]. Non-O157 STEC infections are likely to be under-reported due to

awareness and difficulties in isolation and identification in clinical laboratories.

STEC possesse a number of virulence factors, with the production of Shiga toxins (Stxs) being the most critical which leads to the damage of the endothelial cells and potential HUS [13]. The Stx family can be categorized into two major types, Stx1 and Stx2 [14], which differ in their effects on the endothelial cells [15]. Stx1 and Stx2 are further divided into 3 subtypes (Stx1a, Stx1c and Stx1d) and 7 subtypes (Stx2a to Stx2g) respectively [14]. The different Stx types and/or subtypes may be associated with differences in the severity of illness [16,17]. Other factors are purported to increase virulence in STEC isolates. Cytotoxic necrotizing factor 1 (CNF1) and its isoform CNF2 are cytotoxins that activate Rho GTPases leading to tissue damage, perturb the epithelial barrier and impair the function of immune cells [18]. EAST-1 is a genetically distinct toxin structurally related to heat-stable enterotoxin (STa) of enterotoxigenic *E. coli* [19]. Subtilase cytotoxin (SubAB) is the prototype of a new AB$_5$ toxin family produced by a subset of STEC strains [20]. SubAB is lethal for

mice and induces pathological features overlapping those seen in HUS [21].

Typically STEC also possesse the locus of enterocyte effacement (LEE), which encodes proteins necessary for the formation of attaching and effacing (A/E) lesions including the intimin, a translocated intimin receptor (Tir), a type III secretion apparatus, and effector proteins translocated by the secretion system [22]. In the absence of intimin, other adherence factors may increase adherence and virulence in STEC. These include Iha (IrgA homologue adhesin) which is a STEC adherence-conferring molecule conferring the adherence phenotype upon nonadherent laboratory *E. coli* [23]; Efa1 (EHEC factor for adherence 1) which was shown to be essential for the adherence of the bacteria to cultured epithelial cells, hemagglutination and autoaggregation [24]; LPF (long polar fimbriae) which is closely related to the LPF of *Salmonella enterica* serovar Typhimurium [25]; and Saa (STEC autoagglutinating adhesin) which is an autoagglutinating adhesin produced by LEE-negative STEC strains [26]. Paa (porcine A/E associated protein), which was first discovered in porcine enteropathogenic *E. coli*, contributes to the early stages of the development of the A/E lesions and is also present in O157:H7 [27]. Additionally, many STEC strains contain the heterologous 60-MDa virulence plasmid, which contains a number of virulence genes: an enterohemolysin (*ehxA*), a catalase-peroxidase (*katP*), an extracellular serine protease (*espP*) and a adhesin (*toxB*) [28].

Domestic or wild animals are the primary sources of STEC, such as cattle, pig, sheep, dog, cat, horse, deer and wild boars [29,30,31,32,33], with cattle being regarded as the main natural reservoirs [34]. Humans are the accidental host of STEC through the ingestion of contaminated meat, milk, vegetables, fruits and water. The yak (*Bos grunniens*) lives at high altitude (above 3,000 m) in China, India, Nepal and other countries. There are more than 14 million yaks on the Qinghai-Tibetan plateau, which represent more than 90% of the world yak population. Yaks are adapted to the harsh environments of severe cold, less atmospheric oxygen, strong ultra-violet radiation and poor forage resources. Domestic yaks are of economic importance (such as meat, milk for food, hide for leather and dung for fuel) to Tibetans and other nomadic pastoralists in high-altitude environments [35,36]. In this study we determined the occurrence and characteristics of STEC from yaks from the Qinghai-Tibetan plateau, China.

Materials and Methods

Collection of Samples and Enrichment of Fecal Samples

The investigation was carried out in Yushu tibetan autonomous prefecture, Qinghai province, China. Four big herds (more than one thousand free-ranging yaks) were chosen during June to August, 2012. The sites are Jielachong (3,970 m above msl (mean sea level), latitude of $33°48'$ and longitude of $96°51'$), Gandacun (4,322 m above msl, latitude of $33°13'$ and longitude of $96°73'$), Batangtan (3,987 m above msl, latitude of $32°51'$ and longitude of $96°56'$) and Batang (3,871 m above msl, latitude of $32°84'$ and longitude of $97°11'$) respectively. Fresh fecal samples of yaks were collected in 2 ml sterile tubes containing Luria-Bertani (LB) medium (30% glycerol added). Collected samples were stored at $-20°C$ immediately and transported to the laboratory in National Institute of Communicable Disease Control and Prevention, China CDC in ice cold conditions. A total of 728 fecal samples was collected for the present study.

Each fecal sample was inoculated into modified Tryptone Soya Broth (mTSB) supplemented with novobiocin (10 μg/μl) (Oxoid, UK) and incubated at $37°C$ for 18 to 24 h with shaking at 200 rpm.

stx Screening by TaqMan Real-time PCR

The enriched samples were investigated for stx_1/stx_2 genes by TaqMan duplex real-time PCR assay developed in this study (Probe-1, primers Stx1Fr and Stx1Rr for stx_1; Probe-2, primers Stx2Fr and Stx2Rr for stx_2) (**Table 1**). Briefly, 1.5 ml of each enrichment sample was centrifuged at 13,000×g for 2 min, the pellet was suspended in 150 μl of the rapid lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.3], 1 mM EDTA [pH 9.0], 1% Triton X-100), then boiled for 10 min, and centrifuged at 13,000×g for 2 min. The supernatant was then used as template. Real-time PCR was performed with the Rotor-Gene Q Real-Time PCR system (Qiagen, Germany) using oligonucleotide primers and fluorescent probes targeting stx_1 and stx_2. The amplification conditions were as follows: initial denaturation at $95°C$ for 10 s and then 40 cycles of $95°C$ for 5 s and $60°C$ for 20 s.

The performance of the TaqMan real-time PCR was validated with reference plasmid constructs, pMD18-stx1 and pMD18-stx2, containing a copy of stx_1 and stx_2 respectively and O157:H7 EDL933 spiked human stools. The limit of detection for stx_1 or stx_2 was $1×10^2$ copies per reaction using the reference plasmids and $3.55×10^3$ CFU per gram in spiked human stools. Seventeen non-STEC pathogens were used to evaluate assay specificity, including enteroaggregative *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, *Shigella flexneri*, *Shigella sonnei*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Vibrio cholerae*, *Salmonella typhi*, *Salmonella typhimurium*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Citrobacter freundii* and *Campylobacter jejuni*. No false-positives were observed.

Isolation of Shiga Toxin-producing *Escherichia coli*

Enriched fecal samples tested positive by the TaqMan real-time PCR assay for stx_1 and/or stx_2 genes were plated onto CHROMagar™ ECC agar (CHROMagar, Paris, France), and incubated at $37°C$ overnight. Ten presumptive colonies (blue or colorless, round moist colonies, but the colonial morphology may be variable) on each plate were picked and screened for the presence of stx_1 and/or stx_2 genes by single colony duplex PCR assay (primers Stx1F and Stx1R for stx_1, primers Stx2F and Stx2R for stx_2) (**Table 1**). The *stx*-positive colonies were plated onto LB and incubated overnight to obtain single colonies for further identification. If all 10 colonies were negative for *stx*, another 10 colonies were picked and screened. Finally, 1 to 3 *stx*-positive isolates from each sample were collected for further investigation.

Biochemical Test and Serotyping of STEC Isolates

stx-positive isolates were confirmed to be *E. coli* by biochemical identification using the API 20E system (bioMérieux, France). The O serogroups were screened by PCR using O antigen specific primers in DebRoy *et al.* [37]. *E. coli* O antisera (Statens Serum Institute, Denmark) were used to confirm the O group PCR results. The H type of each isolate was determined by amplifying and sequencing the *fliC* gene and comparing sequences in GenBank as previously described [34].

Identification of Virulence and Adherence Factor Genes

All STEC isolates were subjected to PCR for detection of intimin-encoding gene (*eaeA*), putative adhesin genes (*iha*, *efa1*, $lpfA_{O157/OI-141}$, $lpfA_{O157/OI-154}$, $lpfA_{O113}$, *saa*, *paa*), virulence-associated genes (*cnf1*, *cnf2*, *astA*, *subA*), the large heterologous virulence plasmid genes (*exhA*, *katP*, *espP*, *toxB*) using primers listed in **Table 1**.

Table 1. PCR primers used for the detection of STEC virulence or adherence genes.

Target	Primer	Oligonucleotide sequence (5′-3′)	Amplicon size (bp)	Annealing temperature (°C)	Reference
stx_1	Stx1Fr	TGGATTTAATGTCGCATAGTGGAA	82	60	This study[a]
	Stx1Rr	CAGCTGTCACAGTAACAAACCGTAA			
	Probe-1	HEX-CACTGACGCAGTCTGTGGCAAGAGC-BHQ1			
stx_2	Stx2Fr	CAACGGACAGCAGTTATACCACTCT	103	60	This study[a]
	Stx2Rr	TTAACGCCAGATATGATGAAACCA			
	Probe-2	FAM-CCGGAATGCAAATCAGTCGTCACTCA-BHQ1			
stx_1	Stx1F	AAATCGCCATTCGTTGACTACTTCT	370	58	This study[b]
	Stx1R	TGCCATTCTGGCAACTCGCGATGCA			
stx_2	Stx2F	CAGTCGTCACTCACTGGTTTCATCA	283	58	This study[b]
	Stx2R	GGATATTCTCCCCACTCTGACACC			
stx_1	SltIF	TCGCATGAGATCTGACC	1470	60	[56][c]
	SltIR	AACTGACTGAATTGAGATG			
stx_2	GK1	ATGAAGTGTATATTATTTAAATGG	1260	55	[56][c]
	GK4	TCAGTCATTATTAAACTGCAC			
eaeA	eaeAF	TCAATGCAGTTCCGTTATCAGTT	482	58	[57]
	eaeAR	GTAAAGTCCGTTACCCCAACCTG			
iha	iha-I	CAGTTCAGTTTCGCATTCACC	1305	56	[58]
	iha-II	GTATGGCTCTGATGCGATG			
efa1	efa1F	GAGACTGCCAGAGAAAG	479	51	[24]
	efa1R	GGTATTGTTGCATGTTCAG			
$lpfA_{O157/OI-154}$	OI-154F	GCAGGTCACCTACAGGCGGC	525	55	[59]
	OI-154R	CTGCGAGTCGGCGTTAGCTG			
$lpfA_{O157/OI-141}$	OI-141F	CTGCGCATTGCCGTAAC	412	54	[60]
	OI-141R	ATTTACAGGCGAGATCGTG			
$lpfA_{O113}$	O113F	ATGAAGCGTAATATTATAG	573	52	[61]
	O113R	TTATTTCTTATATTCGAC			
saa	saaF	CGTGATGAACAGGCTATTGC	119	52	[62]
	saaR	ATGGACATGCCTGTGGCAAC			
paa	M155-F1	ATGAGGAAACATAATGGCAGG	350	60	[63]
	M155-R1	TCTGGTCAGGTCGTCAATAC			
cnf1	CNF1-fp	GGCGACAAATGCAGTATTGCTTGG	552	63	[64]
	CNF1-bp	GACGTTGGTTGCGGTAATTTTGGG			
cnf2	CNF2-fp	GTGAGGCTCAACGAGATTATGCACTG	839	63	[64]
	CNF2-bp	CCACGCTTCTTCTTCAGTTGTTCCTC			
astA	EAST11a	CCATCAACACAGTATATCCGA	111	55	[65]
	EAST11b	GGTCGCGAGTGACGGCTTTGT			
subA	SubHCDF	TATGGCTTCCCTCATTGCC	556	65/60	[66]
	SubSCDR	TATAGCTGTTGCTTCTGACG			
ehxA	hlyAF	GGTGCAGCAGAAAAAGTTGTAG	1551	57	[67]
	hlyAR	TCTCGCCTGATAGTGTTTGGTA			
katP	wkat-B	CTTCCTGTTCTGATTCTTCTGG	2125	56	[68]
	wkat-F	AACTTATTTCTCGCATCATCC			
espP	esp-A	AAACAGCAGGCACTTGAACG	1830	56	[28]
	esp-B	GGAGTCGTCAGTCAGTAGAT			
toxB	toxB.911F	ATACCTACCTGCTCTGGATTGA	602	55	[69]
	toxB.1468R	TTCTTACCTGATCTGATGCAGC			

[a]Primers and probes used for TaqMan real-time PCR for the screening of stx_1 and stx_2.
[b]Primers used for duplex PCR for the detection of stx_1 and stx_2.
[c]Primers used for amplifying and sequencing the full length of stx_1 or stx_2.
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stx Subtyping

Genotyping of stx_1 and stx_2 subtypes was conducted by the PCR subtyping method developed by Scheutz et al. [14]. The complete stx_1 and/or stx_2 genes of some STEC isolates were amplified (primers SltIF and SltIR for stx_1, primers GK1 and GK4 for stx_2) (**Table 1**) and sequenced. DNA sequences were then analyzed and compared with the published sequences of stx_1 and stx_2 subtypes in the GenBank.

Pulsed-field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis was performed using the non-O157 STEC subtyping protocol from PulseNet, USA with some modifications. The genomic DNA was digested with 45 U of *Xba*I (Takara, Dalian, China) at 37°C for 2 h. A contour-clamped homogenous electric field apparatus CHEF-Mapper (Bio-Rad, USA) was used. The pulse time was ramped from 6.76 s to 35.38 s over 18 h at 6.0 V/cm. The image was captured with a Gel Documentation 2000 software (Bio-Rad, USA) and exported to Bionumerics (Version 4.0, Applied Maths BVBA, Belgium) for analysis of the PFGE patterns. An UPGMA dendrogram was drawn using the BioNumerics software.

Ethics Statement

Fecal samples of free-ranging yaks were acquired with the consent of the owners of the lands and animals. The study was approved by the ethics committee of National Institute for Communicable Disease Control and Prevention, China CDC, according to the medical research regulations of Ministry of Health, China.

Results

Prevalence of STEC in Yak Fecal Samples

Out of 728 yak fecal samples analyzed in this study, 138 (18.96%) were positive for stx_1 and/or stx_2 genes using TaqMan real-time PCR assay. The four herds showed different stx_1 and/or stx_2 positive rates ranging from 14% to 29%. One hundred and twenty eight STEC isolates were isolated from 85 of the 138 stx positive fecal samples giving a culture positive STEC rate of 61.59% for stx PCR positive samples and 11.68% for all samples (**Table 2**). A single isolate was obtained from 44 fecal samples, two isolates per sample were recovered from 39 fecal samples, and three isolates each were obtained from two samples.

Serogroups and Serotypes

In total, 15 different O serogroups and 12 different H types were identified in the 128 STEC isolates, which belonged to 36 divergent serotypes namely O2:H21, O2:H45, O6:H14, O6:H21, O6:Hnt, O8:H2, O8:H9, O8:H16, O8:H19, O8:H45, O12:H12, O22:H8, O52:H2, O66:H8, O66:H21, O78:H8, O78:H21, O78:H44, O78:H45, O78:Hnt, O12/O78:H44, O117:H2, O117:H21, O123:H8, O127:H8, O137:H21, O149:H45, O158:H8, O158:H16, O165:H8, O165:H21, Ont:H7, Ont:H8, Ont:H21, Ont:H40, Ont:H44. Twenty one isolates were untypable for O groups and 4 isolates were untypable for H antigen as there was no product from the PCR amplification of the *fliC* gene. The predominant serotypes were O8:H16, O2:H45, O117:H21, O78:H8, O8:H9, Ont:H8, Ont:H21, and O78:H45 which consisted of 14 (10.94%), 14 (10.94%), 11 (8.59%), 8 (6.25%), 8 (6.25%), 8 (6.25%), 7 (5.47%), and 6 (4.69%) isolates respectively. Serotypes O117:H2 and O22:H8 were identified in 4 isolates each. Five serotypes contained 3 isolates each and seven serotypes contained 2 isolates each. Fifteen serotypes contained only 1 isolate each (**Table 3**).

Presence of stx Genes and stx Subtypes and Other Virulence Factor Genes

Among the 128 STEC isolates, 33 were tested positive for stx_1 only, 75 for stx_2 only and 20 positive for both stx_1 and stx_2 (**Table 4** and **Table S1**). All of the 53 stx_1-positive STEC isolates were stx_{1a} subtype. Of the 95 stx_2-positive STEC isolates, 5 stx_2 subtypes were identified with 20 isolates of stx_{2a}, 50 of stx_{2b}, 6 of stx_{2c}, 21 of stx_{2d} and 6 of stx_{2g}. Four isolates carried both stx_{2a} and stx_{2b}, and another 4 isolates carried both stx_{2a} and stx_{2c} (**Table 4** and **Table S1**).

Only two (MN1208-22 and MN1208-34) STEC isolates were *eaeA* positive. Of the 7 putative adhesin genes (*iha*, *efa1*, $lpfA_{O157/OI-141}$, $lpfA_{O157/OI-154}$, $lpfA_{O113}$, *saa*, *paa*) screened, *iha*, *efa1*, *saa* and *paa* were present in 87 (67.97%), 2 (1.56%), 66 (51.56%), 7 (5.47%) STEC isolates respectively. The other 3 genes were not detected in any of the isolates. Seven isolates were positive for only one gene (*paa*). Sixty six isolates were positive for both *iha* and *saa*. Two isolates were positive for 4 genes (*eaeA*, *iha*, *efa1* and *saa*). Thirty four isolates were negative for all the adherence –associated genes tested.

Four additional virulence-associated genes (*cnf1*, *cnf2*, *astA*, *subA*) were screened. Thirteen (10.16%) STEC isolates were positive for both *cnf1* and *cnf2*. Twenty four (18.75%) and 44 (34.38%) were positive for *astA* and *subA* respectively. Interestingly, the *subA* gene was present in none of STEC isolates that carried *astA* gene (**Table 3** and **Table S1**). Among the four virulence plasmid genes (*exhA*, *katP*, *espP*, *toxB*) tested, *exhA* and *espP* were present in 66 (51.56%) and 36 (28.13%) STEC isolates respectively. *espP* positive isolates also carried *exhA*. None of the 128 isolates were *katP* or *toxB* positive.

PFGE

The 128 non-O157 STEC isolates were analyzed by PFGE to investigate their genetic relationship. Five isolates failed to produce distinctive patterns. The remaining 123 isolates were divided into

Table 2. Prevalence of Shiga toxin-producing *Escherichia coli* in yaks.

Herd	No. of samples	No. of *stx* positive (%)	No. of samples with STEC isolates (%)	No. of STEC isolates (%)
1	200	28 (14)	23 (11.5)	40 (20)
2	200	58 (29)	31 (15.5)	40 (20)
3	100	15 (15)	3 (3)	5 (5)
4	228	37 (16.23)	28 (12.28)	43 (18.86)
Total	728	138 (18.96)	85 (11.68)	128 (17.58)

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Table 3. Serotypes and virulence factors of Shiga toxin-producing *Escherichia coli* isolates from yaks*.

Serotype	No. of isolates	*stx*		intimin gene	other putative adherence genes				Other virulence -associated genes				Plasmid genes	
		stx_1	stx_2	*eaeA*	*iha*	*efa1*	*saa*	*paa*	*cnf1*	*cnf2*	*astA*	*subA*	*ehxA*	*espP*
O2:H21	1	0	1	0	1	0	1	0	0	0	0	1	1	1
O2:H45	14	14	0	0	0	0	0	0	0	0	14	0	0	0
O6:H14	2	0	2	0	2	0	2	0	0	0	0	2	2	2
O6:H21	2	2	0	0	2	0	2	0	0	0	0	0	2	0
O8:H2	1	0	1	0	1	0	1	0	0	0	0	1	1	0
O8:H9	8	8	8	0	8	0	8	0	0	0	0	0	8	8
O8:H16	14	0	14	0	14	0	0	0	0	0	1	0	0	0
O8:H19	3	3	0	0	3	0	3	0	0	0	0	3	3	3
O8:H45	3	0	3	0	0	0	0	1	0	0	1	0	0	0
O12:H12	1	1	0	0	1	0	1	0	0	0	0	0	1	1
O12:H44	1	0	1	0	0	0	0	0	0	0	1	0	0	0
O22:H8	4	0	4	0	0	0	0	0	0	0	0	0	0	0
O52:H2	3	0	3	0	2	0	0	0	1	1	0	0	0	0
O66:H8	1	1	0	0	1	0	1	0	0	0	0	1	1	1
O66:H21	2	0	2	0	2	0	2	0	0	0	0	2	2	2
O78:H8	8	2	8	0	8	0	8	0	0	0	0	8	8	1
O78:H21	3	0	3	1	3	1	3	0	0	0	0	3	3	3
O78:H44	1	0	1	0	0	0	0	1	0	0	0	0	0	0
O78:H45	6	0	6	0	1	0	1	5	0	0	5	1	1	1
O117:H2	4	0	4	0	4	0	0	0	0	0	0	0	0	0
O117:H21	11	0	11	0	0	0	0	0	11	11	1	0	0	0
O123:H8	1	1	1	0	1	0	1	0	0	0	0	1	1	1
O127:H8	1	1	1	0	1	0	1	0	0	0	0	1	1	1
O137:H21	1	1	1	0	1	0	1	0	0	0	0	0	1	0
O149:H45	2	0	2	0	2	0	2	0	0	0	0	2	2	0
O158:H8	2	0	2	0	2	0	2	0	0	0	0	2	2	0
O158:H16	1	0	1	0	1	0	0	0	0	0	0	0	0	0
O165:H8	3	3	1	0	3	0	3	0	0	0	0	3	3	3
O165:H21	1	1	0	0	1	0	1	0	0	0	0	0	1	0
Ont/Hnt**	23	15	14	1	22	1	22	0	1	1	1	13	23	8
Total	128	53	95	2	87	2	66	7	13	13	24	44	66	36

*None of the 128 isolates were positive for *lpfA*$_{O157/OI-154}$, *lpfA*$_{O157/OI-141}$, *lpfA*$_{O113}$, *katP* or *toxB* positive.
**Ont/Hnt: O or H are not typable, including Ont:H8 (8 isolates), Ont:H21 (7 isolates), Ont:H44 (2 isolates), Ont:Hnt (2 isolates), Ont:H7 (1 isolate), Ont:H40 (1 isolate), O78:Hnt (1 isolate), O6:Hnt (1 isolate).
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67 PFGE patterns (EZKX01001 to EZKX01067) (**Figure 1** and **Table S1**). For the 41 fecal samples with two or three isolates, the multiple isolates for 28 samples showed identical PFGE banding pattern, serotype and virulence gene profile (**Figure 1** and **Table S1**), suggesting that the multiple isolates from the same sample belong to the same STEC strain. However, 10 fecal samples contained isolates with different PFGE patterns including 3 samples (samples 354, 597, and 630) showing different PFGE patterns only (all have the same serotype), 2 samples (samples 6 and 121) showing different PFGE patterns as well as different serotypes, and 5 samples (samples 30, 255, 342, 702 and 716) showing different PFGE patterns, different serotypes and different *stx* subtypes (**Figure 1** and **Table S1**). These data suggest that some yaks were colonized by more than one STEC strain. There were also 3 samples (samples 114, 369, 525) with multiple isolates having the same PFGE type but different serotypes (**Figure 1** and **Table S1**).

The PFGE patterns were used to construct an UPGMA dendrogram (**Figure 1**) which shows that the STEC isolates were genetically diverse with nodes linking single isolates or groups of isolates at less than 80% similarity. Interestingly many isolates were grouped together with similarity at 90% or greater suggesting close genetic relationships. In particular, isolates of the same serotype had the tendency to cluster together and also carried identical *stx* or *stx* subtypes. These includes O117:H21, O8:H9, O8:H16, O2:H45, O22:H8 and O78:H45 carrying stx_{2b}, stx_{1a}+stx_{2d}, stx_{2b}, stx_{1a}, stx_{2b} and stx_{2g} respectively. The O117:H21 isolates were in 3 related nodes while the others were in their own single node. Isolates showing identical PFGE patterns came from the same yak herd with the exception of EZKX01005

Table 4. Summary of *stx* subtyping in 128 STEC isolates.

No. of isolates	*stx* subtype					
	stx$_{1a}$	*stx*$_{2a}$	*stx*$_{2b}$	*stx*$_{2c}$	*stx*$_{2d}$	*stx*$_{2g}$
44	–	–	+	–	–	–
33	+	–	–	–	–	–
15	+	–	–	–	+	–
11	–	+	–	–	–	–
6	–	–	–	–	+	–
6	–	–	–	–	–	+
4	–	+	–	+	–	–
2	+	–	+	–	–	–
2	–	+	+	–	–	–
2	–	–	–	+	–	–
2	+	+	+	–	–	–
1	+	+	–	–	–	–
128	53	20	50	6	21	6

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which contained 5 isolates from herd 2 and 1 isolate from herd 4. The herd 4 isolate (MN1287-15) also shared the same H antigen, *stx*$_{1a}$ and the presence of *iha*, *saa* and *ehxA* as the herd 2 isolates. However this PFGE type displayed higher heterogeneity with 2 different O and 2 different H antigens and 1 isolate also carrying *astA*. The two isolates from another 2 samples (samples 114 and 525) each showed the same PFGE type but different serotypes.

The virulence gene profiles also showed a clustered distribution but less pronounced than the serotypes and *stx* subtypes. The main nodes containing the following serotypes were uniformly positive for some of the virulence factors: O117:H21 was positive for both and only *cnf1* and *cnf2*; O2:H45 for *astA* only, O8:H9 for *iha*, *saa*,*ehxA* and *espA*; O8:H16 for *iha* only; O78:H8 for *iha*, *saa*, *subA* and *ehxA*; and O78:H45 for both *paa* and *astA*. Interestingly O22:H8 carried none of the virulence factors tested (**Table S1**).

Discussion

Ruminants, especially cattle, are the major reservoirs of STEC. The prevalence of STEC in beef cattle ranged from 0.2 to 27.8% for O157 STEC, and 2.1 to 70.1% for non-O157 STEC [38]. Bandyopadhyay et al. [39] recovered 42 STEC isolates from 273 rectal swab samples (15.38%) in an STEC study of the yak *Poephagus grunniens*. Our results showed a similar rate of STEC isolation. Of the 728 yak (*Bos grunniens*) fecal samples screened, 18.96% of the samples (138/728) were positive for the *stx* genes by PCR and 11.68% (85/728) by culture. Interestingly nearly 40% of the STEC positive samples by PCR were negative by culture. It seems that either the STEC cell numbers were low in the fecal sample or *stx*-positive non-*E. coli* was present in the feces. Bosilevac et al. [34] screened ground beef samples for STEC and only recovered STEC by culture from 300 out of the 1006 *stx*-positive samples with a success rate of just 30%.

More than 435 STEC serotypes have been recovered from cattle. Serotypes O8:H2, O8:H9, O8:H16, O8:19, O22:H8, O117:H2, O165:H8 found in our study were also reported in cattle, beef, meat and milk product [34,40,41,42]. Three serotypes, O8:H2, O8:H19 and O22:H8, have been isolated from human infections [40]. The more common HUS-causing serotypes, such as O157:H7, O26:H11, O103:H2, O111:NM, O121:H19 and O145:NM [43] were not isolated from the yaks. Neither was STEC O104:H4, the cause of the 2011 outbreaks in Germany and France. This finding is in agreement with the failure to find this isolate in cattle [44,45] and gives an additional evidence that ruminants are not reservoirs of the outbreak isolate.

Bandyopadhyay et al. reported the occurrence and characteristics of STEC from feces, milk and milk products of *Poephagus grunniens*, another species of the yaks, in India [39,46,47]. The STEC strain isolated from the feces of *Poephagus grunniens* belonged to 22 divergent O serogroups [39]. Among these serogroups, only three (O2, O22 and O158) were also present in our study, suggesting that there are diverse STEC strains of overlapping O serogroups present in these two species of the yaks.

Since the carriage of combinations of the *stx* genes and *stx* subtypes has been associated with disease severity, the profile of the *stx* genes gives us an overview of the pathogenic potential of these STEC isolates from the yaks. In this study, 1 *stx*$_1$ subtype,5 *stx*$_2$ subtypes and 12 different combinations of *stx*$_1$/*stx*$_2$ subtypes were found in the 128 STEC isolates analyzed (**Table 4**). Manning et al. found that clade 8 O157:H7 strains which were significantly more likely to infect patients with HUS and more likely to have both the *stx*$_{2a}$ and *stx*$_{2c}$ genes, implying that the carriage of both the Stx2a and Stx2c phages contributes in part to the greater virulence of clade 8 strains [48]. Mellmann et al. analyzed a collection of 524 EHEC isolated from HUS patients and found that 169 (32.3%) belonged to 34 non-O157 serotypes and that profiles of *stx*$_{1a}$ only, *stx*$_{1c}$ only, *stx*$_{1a}$+*stx*$_{2a}$, *stx*$_{1c}$+*stx*$_{2a}$, *stx*$_{1c}$+*stx*$_{2d}$, *stx*$_{2a}$ only, *stx*$_{2c}$ only, *stx*$_{2d}$ only and *stx*$_{2a}$+*stx*$_{2c}$ were present in these non-O157 STEC isolates [2]. In our STEC isolates, several of above profiles were also present including *stx*$_{1a}$ only, *stx*$_{1a}$+*stx*$_{2a}$, *stx*$_{2a}$, *stx*$_{2c}$ only, *stx*$_{2d}$ only and *stx*$_{2a}$+*stx*$_{2c}$. These results suggest that the non-O157 STEC isolates from the yaks have the potential to cause human illness and there is a need to monitor the local human population for STEC infections.

Non–O157 STEC isolates that carry both *stx*$_2$ and *eae* genes were more often associated with severe disease [49,50]. In this study only 2 *eae* positive isolates (MN1208-22 and MN1208-34) were isolated from the yaks, both of which carried *stx*$_{2a}$, indicating their virulence potential. The 2 isolates were on the same PFGE node, but interspersed by other *eae* negative isolates (**Figure 1**), suggesting independent acquisition of the LEE locus by the two isolates. However, although *eae* encoded on the LEE pathogenicity island is absent from almost all of the STEC isolates, non-LEE-encoded effector proteins potentially involved in virulence have been demonstrated in some serotypes identified in this study [51].

Since the majority of the STEC isolates were *eae* negative, we investigated other factors associated with adherence including Iha and Saa, both of which have been reported to be correlated with increased adherence in the *eae* negative strains [23,26,52]. We found that a high proportion of the yak STEC isolates contained *iha* (67.97%) and *saa* (51.56%). This finding is similar to that reported by Bosilevac et al. [34] in the cattle where 88% and 73% of the STEC isolates were positive for *iha* and *saa* respectively. The study of Bosilevac et al. further found that the *saa* gene was always present in the absence of *eae* and was more often present in isolates that were also positive for the large virulence plasmid [34]. Our yak STEC isolates also showed a similar correlation. Interestingly our two *eae* positive isolates also harbored the *iha* and *saa* genes.

Of the two other adhesin genes, *lpfA* and *paa* screened, all 128 isolates were negative for *lpfA* while only 7 isolates were positive for *paa*, 5 of which belonged to the same serotype (O78:H45). Thirty four isolates were negative for all the adherence –associated genes tested (*eae*, *iha*, *efa1*, *lpfA*$_{O157/OI-154}$, *lpfA*$_{O157/OI-141}$, *lpfA*$_{O113}$, *saa*,

Dice (Opt:1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]

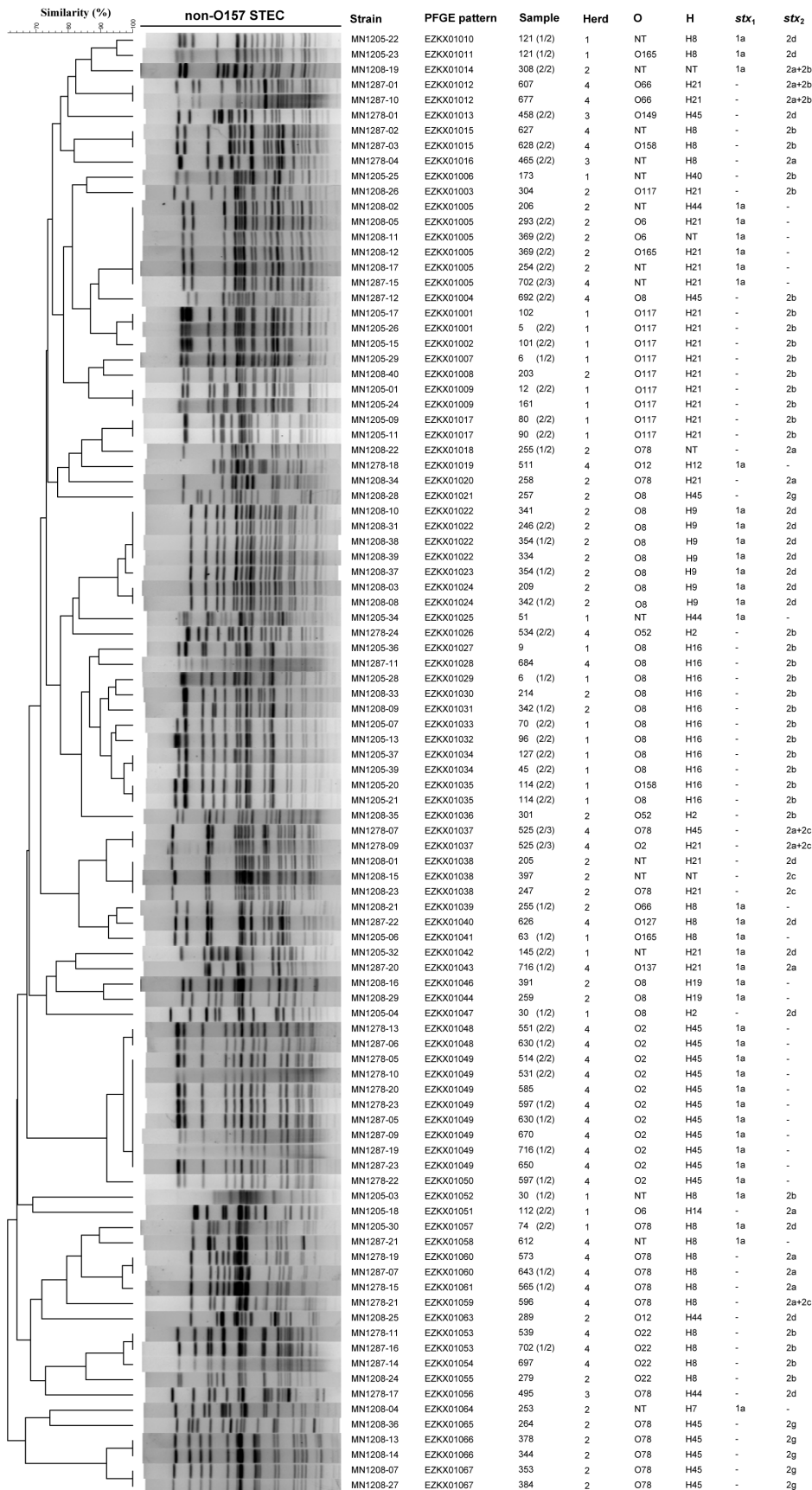|	Strain	PFGE pattern	Sample	Herd	O	H	stx_1	stx_2
	MN1205-22	EZKX01010	121 (1/2)	1	NT	H8	1a	2d
	MN1205-23	EZKX01011	121 (1/2)	1	O165	H8	1a	2d
	MN1208-19	EZKX01014	308 (2/2)	2	NT	NT	1a	2a+2b
	MN1287-01	EZKX01012	607	4	O66	H21	-	2a+2b
	MN1287-10	EZKX01012	677	4	O66	H21	-	2a+2b
	MN1278-01	EZKX01013	458 (2/2)	3	O149	H45	-	2d
	MN1287-02	EZKX01015	627	4	NT	H8	-	2b
	MN1287-03	EZKX01015	628 (2/2)	4	O158	H8	-	2b
	MN1278-04	EZKX01016	465 (2/2)	3	NT	H8	-	2a
	MN1205-25	EZKX01006	173	1	NT	H40	-	2b
	MN1208-26	EZKX01003	304	2	O117	H21	-	2b
	MN1208-02	EZKX01005	206	2	NT	H44	1a	-
	MN1208-05	EZKX01005	293 (2/2)	2	O6	H21	1a	-
	MN1208-11	EZKX01005	369 (2/2)	2	O6	NT	1a	-
	MN1208-12	EZKX01005	369 (2/2)	2	O165	H21	1a	-
	MN1208-17	EZKX01005	254 (2/2)	2	NT	H21	1a	-
	MN1287-15	EZKX01005	702 (2/3)	4	NT	H21	1a	-
	MN1287-12	EZKX01004	692 (2/2)	4	O8	H45	-	2b
	MN1205-17	EZKX01001	102	1	O117	H21	-	2b
	MN1205-26	EZKX01001	5　(2/2)	1	O117	H21	-	2b
	MN1205-15	EZKX01002	101 (2/2)	1	O117	H21	-	2b
	MN1205-29	EZKX01007	6　(1/2)	1	O117	H21	-	2b
	MN1208-40	EZKX01008	203	2	O117	H21	-	2b
	MN1205-01	EZKX01009	12　(2/2)	1	O117	H21	-	2b
	MN1205-24	EZKX01009	161	1	O117	H21	-	2b
	MN1205-09	EZKX01017	80　(2/2)	1	O117	H21	-	2b
	MN1205-11	EZKX01017	90　(2/2)	1	O117	H21	-	2b
	MN1208-22	EZKX01018	255 (1/2)	2	O78	NT	-	2a
	MN1278-18	EZKX01019	511	4	O12	H12	1a	-
	MN1208-34	EZKX01020	258	2	O78	H21	-	2a
	MN1208-28	EZKX01021	257	2	O8	H45	-	2g
	MN1208-10	EZKX01022	341	2	O8	H9	1a	2d
	MN1208-31	EZKX01022	246 (2/2)	2	O8	H9	1a	2d
	MN1208-38	EZKX01022	354 (1/2)	2	O8	H9	1a	2d
	MN1208-39	EZKX01022	334	2	O8	H9	1a	2d
	MN1208-37	EZKX01023	354 (1/2)	2	O8	H9	1a	2d
	MN1208-03	EZKX01024	209	2	O8	H9	1a	2d
	MN1208-08	EZKX01024	342 (1/2)	2	O8	H9	1a	2d
	MN1205-34	EZKX01025	51	1	NT	H44	1a	-
	MN1278-24	EZKX01026	534 (2/2)	4	O52	H2	-	2b
	MN1205-36	EZKX01027	9	1	O8	H16	-	2b
	MN1287-11	EZKX01028	684	4	O8	H16	-	2b
	MN1205-28	EZKX01029	6　(1/2)	1	O8	H16	-	2b
	MN1208-33	EZKX01030	214	2	O8	H16	-	2b
	MN1208-09	EZKX01031	342 (1/2)	2	O8	H16	-	2b
	MN1205-07	EZKX01033	70　(2/2)	1	O8	H16	-	2b
	MN1205-13	EZKX01032	96　(2/2)	1	O8	H16	-	2b
	MN1205-37	EZKX01034	127 (2/2)	1	O8	H16	-	2b
	MN1205-39	EZKX01034	45　(2/2)	1	O8	H16	-	2b
	MN1205-20	EZKX01035	114 (2/2)	1	O158	H16	-	2b
	MN1205-21	EZKX01035	114 (2/2)	1	O8	H16	-	2b
	MN1208-35	EZKX01036	301	2	O52	H2	-	2b
	MN1278-07	EZKX01037	525 (2/3)	4	O78	H45	-	2a+2c
	MN1278-09	EZKX01037	525 (2/3)	4	O2	H21	-	2a+2c
	MN1208-01	EZKX01038	205	2	NT	H21	-	2d
	MN1208-15	EZKX01038	397	2	NT	NT	-	2c
	MN1208-23	EZKX01038	247	2	O78	H21	-	2c
	MN1208-21	EZKX01039	255 (1/2)	2	O66	H8	1a	-
	MN1287-22	EZKX01040	626	4	O127	H8	1a	2d
	MN1205-06	EZKX01041	63　(1/2)	1	O165	H8	1a	-
	MN1205-32	EZKX01042	145 (2/2)	1	NT	H21	1a	2d
	MN1287-20	EZKX01043	716 (1/2)	4	O137	H21	1a	2a
	MN1208-16	EZKX01046	391	2	O8	H19	1a	-
	MN1208-29	EZKX01044	259	2	O8	H19	1a	-
	MN1205-04	EZKX01047	30　(1/2)	1	O8	H2	-	2d
	MN1278-13	EZKX01048	551 (2/2)	4	O2	H45	1a	-
	MN1287-06	EZKX01048	630 (1/2)	4	O2	H45	1a	-
	MN1278-05	EZKX01049	514 (2/2)	4	O2	H45	1a	-
	MN1278-10	EZKX01049	531 (2/2)	4	O2	H45	1a	-
	MN1278-20	EZKX01049	585	4	O2	H45	1a	-
	MN1278-23	EZKX01049	597 (1/2)	4	O2	H45	1a	-
	MN1287-05	EZKX01049	630 (1/2)	4	O2	H45	1a	-
	MN1287-09	EZKX01049	670	4	O2	H45	1a	-
	MN1287-19	EZKX01049	716 (1/2)	4	O2	H45	1a	-
	MN1287-23	EZKX01049	650	4	O2	H45	1a	-
	MN1278-22	EZKX01050	597 (1/2)	4	O2	H45	1a	-
	MN1205-03	EZKX01052	30　(1/2)	1	NT	H8	1a	2b
	MN1205-18	EZKX01051	112 (2/2)	1	O6	H14	-	2a
	MN1205-30	EZKX01057	74　(2/2)	1	O78	H8	1a	2d
	MN1287-21	EZKX01058	612	4	NT	H8	1a	-
	MN1278-19	EZKX01060	573	4	O78	H8	-	2a
	MN1287-07	EZKX01060	643 (1/2)	4	O78	H8	-	2a
	MN1278-15	EZKX01061	565 (1/2)	4	O78	H8	-	2a
	MN1278-21	EZKX01059	596	4	O78	H8	-	2a+2c
	MN1208-25	EZKX01063	289	2	O12	H44	-	2d
	MN1278-11	EZKX01053	539	4	O22	H8	-	2b
	MN1287-16	EZKX01053	702 (1/2)	4	O22	H8	-	2b
	MN1287-14	EZKX01054	697	4	O22	H8	-	2b
	MN1208-24	EZKX01055	279	2	O22	H8	-	2b
	MN1278-17	EZKX01056	495	3	O78	H44	-	2d
	MN1208-04	EZKX01064	253	2	NT	H7	1a	-
	MN1208-36	EZKX01065	264	2	O78	H45	-	2g
	MN1208-13	EZKX01066	378	2	O78	H45	-	2g
	MN1208-14	EZKX01066	344	2	O78	H45	-	2g
	MN1208-07	EZKX01067	353	2	O78	H45	-	2g
	MN1208-27	EZKX01067	384	2	O78	H45	-	2g

Figure 1. PFGE profiles of non-O157 STEC isolates from the yaks. The corresponding isolate names, PFGE patterns, no. of samples and herds, serotypes and stx_1 and/or stx_2 subtypes are listed on the right. For samples with more than 1 isolates, the numbers (x/y) in brackets in the sample column are number of strains (x) belonging to that PFGE pattern out of the total number of y isolates from that sample. Note that 5 isolates failed to produce a PFGE pattern and were not on the tree. For full list of isolates and their PFGE patterns and other data, see Table S1.
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paa and *toxB*), suggesting that other novel adherence genes must exist in these STEC isolates, which warrants further investigation.

Of the additional putative virulence factors (*cnf1*, *cnf2*, *astA*, *subA*) screened, the prevalence of *cnf1* and *cnf2* was low (10.16%), with both being present in the same 13 STEC isolates. The *astA* gene was also present at a low frequency of 18.75%. However, the *subA* gene was more prevalent with 34.38% isolates positive. Interestingly, the *subA* gene was present in none of STEC isolates that harbored the *astA* gene. The significance of this mutually exclusive presence is unknown. The prevalence of *subA* in the yaks is similar to that observed in some cattle populations [53]. In contrast, a much smaller proportion of the STEC isolates isolated from human infections were positive for the *subAB* genes, which ranged from 2% in the USA to 10% in Australia [54].

Of the 4 60-MDa large plasmid encoded virulence genes [55], *ehxA*, *katP*, *espP* and *toxB* screened, none of the STEC isolates carried *katP* and *toxB*, whereas 51.56% and 28.13% STEC isolates were positive for *ehxA* and *espP* respectively. All STEC isolates that were positive for *espP* were also positive for *ehxA*. Our findings are similar to that in the cattle population based on isolation from ground beef in which *ehxA* and *espP* were more commonly present [34].

PFGE analysis showed that there is quite high genetic diversity of the STEC in the yaks and it seems that there is no separation of the STEC population between the yak herds. Eight (O8:H16, O8:H45, O22:H8, O52:H2, O78:H8, O78:H21, O78:H45 and O117:H21) of the 36 serotypes were present in 2 different herds.

The serotype data also suggested that some clones are wide spread. The yaks may harbor more than one type of STEC isolates since 11.76% (10/85) of the samples contained 2 or more isolates of different PFGE patterns.

In conclusion, this study was the first report on the occurrence and characteristics of STEC isolated from yaks (*Bos grunniens*). We isolated 128 STEC isolates of different serotypes, *stx* subtypes and virulence gene profiles from the yaks (*Bos grunniens*) from the Qinghai-Tibetan plateau, revealing that *Bos grunniens* are natural reservoirs of STEC. This study further extends our knowledge of the genetic diversity and reservoir host range of STEC. The serotypes and *stx* subtypes identified were partially reported in human infections, pointing to the potential of these STEC isolates to cause disease in humans. Further investigations are needed to assess their public health significance in Tibetans and other nomadic pastoralists in this region.

Supporting Information

Table S1 Profiles of the 128 STEC isolates.
(XLS)

Author Contributions

Conceived and designed the experiments: JX Y. Xiong. Performed the experiments: XB AZ QM DJ BY HS Y. Xiong. Analyzed the data: XB RL Y. Xiong. Contributed reagents/materials/analysis tools: Y. Xin HX SL. Wrote the paper: XB RL Y. Xiong.

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