**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 stx1 and stx2 genes by TaqMan real-time PCR after the sample was enriched in modified Tryptone Soya Broth. Of the 138 (18.96%) stx1 and/or stx2-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 stx1 subtype (stx1a) and 5 stx2 subtypes (stx2a, stx2b, stx2c, stx2d and stx2g) were present in these STEC isolates. Apart from lpfA*O157:H7*, astA, espP, saa, cnf1, cin2, astA, astC, eaeA, iha, fepA, cdtA, cdtB, eal, espP and KatP 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.

**Introduction**

Shiga toxin-producing *Escherichia coli* (STEC) are recognized globally as major foodborne pathogens. Clinical manifestations of STEC infections in humans range from non-bloody diarrhea to 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 possess 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 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 possess the locus of enteroctye 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 fimbiae) 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 (vtxB) [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 are infected by STEC [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 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.

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 flaC 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, iha, lpfA, 157/O1:135, lpfA, 157/O1:135, saa, paa), virulence-associated genes (cnf1, cnf2, astA, subA), the large heterologous virulence plasmid genes (ehxA, katP, espP, toxB) using primers listed in Table 1.
| Target | Primer | Oligonucleotide sequence (5’-3’) | Amplicon size (bp) | Annealing temperature (°C) | Reference |
|--------|--------|----------------------------------|------------------|-----------------|-----------|
| stx1   | Stx1Fr | TGGATTAAATGTCGCGGATGTTGGA         | 82               | 60              | This study⁴ |
|        | Stx1R  | CACGTGTCACGTAACAAACGCTAA          |                  |                 |           |
|        | Probe-1| HEX-CAGTGGACGACTCTGCGGACAGC-BHQ1  |                  |                 |           |
| stx2   | Stx2Fr | CAACGGACGACGTTACCTACACTCT         | 103              | 60              | This study⁴ |
|        | Stx2R  | TTAAGCCGAGATGGATGAACCA             |                  |                 |           |
|        | Probe-2| FAM-CCGGAATACAAATCGTGACCTCA-BHQ1  |                  |                 |           |
| stx1   | Stx1F  | AAATCGCCATGCTGGGATAGTCT           | 370              | 58              | This study⁵ |
|        | Stx1R  | TGCCATTCTGGCAACTCGGCCAGTGC       |                  |                 |           |
| stx2   | Stx2F  | CAGTCGCCACTCTTTGCTTCATCA          | 283              | 58              | This study⁵ |
|        | Stx2R  | GGATATCTCCACCCACCTGACACC         |                  |                 |           |
| stx1   | St1F   | TCGCATGAGATCTGACC                 | 1470             | 60              | [56]      |
|        | St1R   | AAAGGCAGATTGAGATG                 |                  |                 |           |
| stx2   | G1      | ATGAGATGATATATATATATGGA           | 1260             | 55              | [56]      |
|        | G4      | TCACTGGTTATAACCTGAC               |                  |                 |           |
| eaeA   | eaeAF   | TCAATGCGAGGTCTGAGTCCCAG         | 482              | 58              | [57]      |
|        | eaeAR   | GTAAAGTGGTAGCTACCCCAACTG          |                  |                 |           |
| iha    | iha-i   | CAGTGCTGAGTCTGAGGAGAAG           | 1305             | 56              | [58]      |
|        | iha-II  | GTATGGCTCTGTGAGTGCAGTG           |                  |                 |           |
| efa1   | efa1F   | GAGACTGCGAGGGAAG                 | 479              | 51              | [24]      |
|        | efa1R   | GTATGCTGACGTGACTGAG              |                  |                 |           |
| pofA   | pofA-O154| CGACGATGACCTACAGGCGGC           | 525              | 55              | [59]      |
|        | pofA-O154| CTGCGAGATGGCGGTTAGCTG          |                  |                 |           |
| pofA   | pofA-O141| CTGCGATGACGCGCGCAAAC           | 412              | 54              | [60]      |
|        | pofA-O141| CTTCATGACGCGGTTAGCTG          |                  |                 |           |
| pofA   | pofA-O113| O113F ATGAGGCTAATATTAG         | 573              | 52              | [61]      |
|        | pofA-O113| O113R TTATGTTATATTGCAG         |                  |                 |           |
| saa    | saaF   | CGTATGAGCAGCCTATTG              | 119              | 52              | [62]      |
|        | saaR   | ATGACGATGCGGTTGGCAAC            |                  |                 |           |
| paa    | paaF   | ATGAGGAAACATAATGGCCAGG          | 350              | 60              | [63]      |
|        | paaR   | TCTGGTACGCTGCGAATAC             |                  |                 |           |
| cnf1   | CNF1-fp| GCGGATAAATGCATTGCTTTG           | 552              | 63              | [64]      |
|        | CNF1-bp| GACGTCGGTTGGCGTAAATTTGGG         |                  |                 |           |
| cnf2   | CNF2-fp| GGTAGGGCTCAAGGAGATTGCAGCTG       | 839              | 63              | [64]      |
|        | CNF2-bp| CCACGCTTCTCTGAGTGTCCCT           |                  |                 |           |
| astA   | ast1    | CCATCAACAGGATATCGG             | 111              | 55              | [65]      |
|        | ast11b  | CCAAGCTGCTGAGATGG               |                  |                 |           |
| subA   | SubHDCF| TATGGTCTTCTCCATTGCGC           | 556              | 65/60           | [66]      |
|        | SubSCDF| TTAGAGCTGCTTGCTTGACG            |                  |                 |           |
| ehuA   | ehuAF   | GGTGCAGGAGAAAAAGCTTGAG          | 1551             | 57              | [67]      |
|        | ehuAR   | TCTCGCCCTGAATGGTTTTGTA          |                  |                 |           |
| katP   | kat-B   | CTCTCGGTACGTTCCCTG              | 2125             | 56              | [68]      |
|        | kat-F   | AACCTTATCTGACTGACATCC           |                  |                 |           |
| espP   | esp-A   | AAACAGGAGCGCATTGGAAG            | 1830             | 56              | [28]      |
|        | esp-B   | GGAATCGCTGACGTGACTGAT            |                  |                 |           |
| toxB   | toxB-911F| ATACCTACCTGTGCTGATTGA          | 602              | 55              | [69]      |
|        | toxB-1468R| TTCTATCTGATGCTGAC           |                  |                 |           |

*Primers and probes used for TaqMan real-time PCR for the screening of stx1 and stx2.
†Primers used for duplex PCR for the detection of stx1 and stx2.
‡Primers used for amplifying and sequencing the full length of stx1 or stx2.

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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 XbaI (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 \(stx_2\) positive fecal samples giving a culture positive STEC rate of 61.59% for \(stx_2\) 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:H1t, O6: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, O153: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 \(\beta\) 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%), 7 (5.47%), and 6 (4.69%) isolates respectively. Seventeen 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 \(stx_2e\). 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 \(exc\) positive. Of the 7 putative adhesin genes (\(eaeA\), \(fliC\), \(fliA1\), \(fliA157\), \(fliA154\), \(fliA151\), \(saa\), \(paa\)) screened, \(eaeA\), \(fliC\), \(saa\) 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 \(saa\) and \(saa\). Thirty four isolates were negative for all the adherence-associated genes tested.

Four additional virulence-associated genes (\(cya\), \(cyn\), \(astA\), \(subA\)) were screened. Thirty (10.16%) STEC isolates were positive for both \(cya\) and \(cyn\). 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 (\(exa\), \(katP\), \(espP\), \(toxB\)) tested, \(exa\) and \(espP\) were present in 66 (51.56%) and 36 (29.13%) STEC isolates respectively; \(espP\) positive isolates also carried \(exa\). 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
67 PFGE patterns (EZKX01001 to EZKX01067) ([Figure 1 and Table S1](#pone.0065537.t003)). 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](#pone.0065537.g001) and [Table S1](#pone.0065537.t003)), 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](#pone.0065537.g001) and [Table S1](#pone.0065537.t003)). 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](#pone.0065537.g001) and [Table S1](#pone.0065537.t003)).

The PFGE patterns were used to construct an UPGMA dendrogram ([Figure 1](#pone.0065537.g001)) 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, O8:H21, O2:H45 and O78:H45 carrying stx2b, stx1a+stx2d, stx2b, stx1a, stx2b and stx2g 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 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 |
|----------|----------------|-----|--------------|-------------------------------|---------------------------------|---------------|
| O2:H21   | 1              | 0   | 1            | 0                             | 1                               | 1             |
| O2:H45   | 14             | 14  | 0            | 0                             | 0                               | 0             |
| O6:H14   | 2              | 0   | 2            | 0                             | 0                               | 0             |
| O6:H21   | 2              | 2   | 0            | 0                             | 0                               | 0             |
| O8:H2    | 1              | 0   | 1            | 0                             | 0                               | 1             |
| O8:H9    | 8              | 8   | 0            | 0                             | 0                               | 8             |
| O8:H16   | 14             | 0   | 14           | 0                             | 0                               | 0             |
| O8:H19   | 3              | 3   | 0            | 3                             | 0                               | 3             |
| O8:H45   | 3              | 0   | 3            | 0                             | 0                               | 0             |
| O12:H12  | 1              | 1   | 0            | 1                             | 0                               | 1             |
| O12:H44  | 1              | 0   | 1            | 0                             | 0                               | 0             |
| O22:H8   | 4              | 0   | 4            | 0                             | 0                               | 0             |
| O52:H2   | 3              | 0   | 3            | 0                             | 2                               | 1             |
| O66:H8   | 1              | 1   | 0            | 1                             | 0                               | 1             |
| O66:H21  | 2              | 0   | 2            | 0                             | 0                               | 0             |
| O78:H8   | 8              | 2   | 8            | 0                             | 0                               | 8             |
| O78:H21  | 3              | 0   | 3            | 1                             | 3                               | 3             |
| O78:H44  | 1              | 0   | 1            | 0                             | 0                               | 0             |
| O78:H45  | 6              | 0   | 6            | 0                             | 1                               | 5             |
| O117:H2  | 4              | 0   | 4            | 0                             | 0                               | 0             |
| O117:H21 | 11             | 0   | 0            | 0                             | 11                              | 11            |
| O123:H8  | 1              | 1   | 1            | 0                             | 0                               | 1             |
| O127:H8  | 1              | 1   | 1            | 0                             | 0                               | 1             |
| O137:H21 | 1              | 1   | 1            | 0                             | 0                               | 1             |
| O149:H45 | 2              | 0   | 2            | 0                             | 0                               | 2             |
| O158:H8  | 2              | 0   | 2            | 0                             | 0                               | 2             |
| O158:H16 | 1              | 0   | 1            | 0                             | 0                               | 0             |
| O165:H8  | 3              | 3   | 1            | 0                             | 0                               | 3             |
| O165:H21 | 1              | 0   | 1            | 0                             | 0                               | 1             |
| Ont/Hnt**| 23             | 15  | 14           | 1                             | 22                              | 11            |

*None of the 128 isolates were positive for lpfA*<sub>O157</sub>*,lpfA*<sub>O157</sub>*<sub>141</sub>, *lpfA*<sub>O113</sub>, *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), Ont:Hnt (1 isolate). doi:10.1371/journal.pone.0065537.t003
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\textsubscript{1a}, and the presence of iha, saa, and espA 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 ecf1 and ecf2; O2:H45 for astA only, O8:H9 for iha, saa, espA and espD; O8:H16 for iha only; O78:H8 for iha, saa, subA, and espA; and O78:H5 for both paa and astA. Interestingly O22:H6 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]. Bandopadhyay et al. [39] recovered 42 STEC isolates from 275 rectal swab samples (15.38%) in an STEC study of the yak Poephagus grunniens. Their 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-\textit{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 455 STEC serotypes have been recovered from cattle. Serotypes O8:H2, O8:H9, O8:H16, O8:19, O22:H8, O117:H2, O115:H5 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.

Bandopadhyay et al. reported the occurrence and characteristics of STEC from feces, milk and milk products of \textit{Poephagus grunniens}, another species of the yaks, in India [39,46,47]. The STEC strain isolated from the feces of \textit{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\textsubscript{1a} subtype, 5 stx\textsubscript{2b} subtypes and 12 different combinations of stx\textsubscript{1a}/stx\textsubscript{2b} 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\textsubscript{2a} and stx\textsubscript{2c} genes, implying that the carriage of both the Stx\textsubscript{2a} and Stx\textsubscript{2c} 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\textsubscript{2a} only, stx\textsubscript{2a} only, stx\textsubscript{1a}+stx\textsubscript{2a}, stx\textsubscript{1a}+stx\textsubscript{2b}, stx\textsubscript{1a}+stx\textsubscript{2a}+stx\textsubscript{2b} only, stx\textsubscript{2a} only, stx\textsubscript{2b} only and stx\textsubscript{2a}+stx\textsubscript{2b} were present in these non-O157 STEC isolates [2]. In our STEC isolates, several of above profiles were also present including stx\textsubscript{1a} only, stx\textsubscript{1a}+stx\textsubscript{2a} only, stx\textsubscript{2a} only and stx\textsubscript{2a}+stx\textsubscript{2b}. 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\textsubscript{2a} 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\textsubscript{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 screened, all 128 STEC isolates from the yaks. In this study, 1 iha positive isolates (MN1208-22 and MN1208-34) were isolated from the yaks, both of which carried stx\textsubscript{2a}, indicating their virulence potential. The 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].

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

| No. of isolates | stx\textsubscript{1a} | stx\textsubscript{2a} | stx\textsubscript{2b} | stx\textsubscript{2c} | stx\textsubscript{2d} | stx\textsubscript{2g} |
|----------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| 128            | 53                  | 20                  | 50                  | 6                   | 21                  | 6                   |

| doi:10.1371/journal.pone.0065537.t004 |
The corresponding isolate names, PFGE patterns, no. of samples and herds, serotypes and stx1, and/or stx2 subtypes are listed on the right. For samples with more than 1 isolates, the numbers (x/y) in brackets in the sample columns denote the 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. doi:10.1371/journal.pone.0065537.g001

Figure 1. PFGE profiles of non-O157 STEC isolates from the yaks. 

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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