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PREVALENCE OF SULFONAMIDE AND FLORFENICOL RESISTANCE GENES IN *ESCHERICHIA COLI* ISOLATED FROM YAKS (*BOS GRUNNIENS*) AND HERDSMEN IN THE TIBETAN PASTURE

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ABSTRACT: To determine the antimicrobial susceptibility profiles and prevalence of resistance genes in *Escherichia coli* isolated from yaks (*Bos grunniens*) and herdsmen in nine plateau pastures in Tibet, we isolated 184 nonidentical strains of *E. coli* from yaks and herdsmen. Antimicrobial susceptibility testing of 15 antimicrobials was conducted and the prevalence of sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) and florfenicol resistance genes (*floR*, *cfr*, *cmlA*, *fexA*, *pexA*, and *estDL136*) was determined. *Escherichia coli* isolated from yaks had a high resistance rate to sulfamethoxazole (44%), sulphafurazole (40.4%), and florfenicol (11.4%). *Escherichia coli* isolated from herdsmen had a high resistance rate to sulfamethoxazole (57%) and sulphafurazole (51%). In addition, *sul* genes were present in 93% of sulfonamide-resistant isolates (84/90), and 17 *floR* genes and four *cmlA* genes were found in 19 florfenicol-resistant isolates. Even though florfenicol is prohibited from use in humans, three *floR* genes were detected in strains isolated from herdsmen. The three *floR*-positive isolates from herdsmen had pulsed-field gel electrophoresis patterns similar to isolates from yaks. In addition to documenting the *sul* and *floR* genes in *E. coli* isolated from yaks and herdsmen in the Tibetan pasture, we demonstrated the potential risk that antimicrobial-resistant *E. coli* could spread among herdsmen and yaks.

Key words: Dulfonamides, *Escherichia coli*, florfenicol, herdsmen, yaks.

INTRODUCTION

Yaks (*Bos grunniens*) are mammals adapted to high-altitude and extremely cold conditions. They provide an important source of milk, meat, fiber, and fuel, making them vital for the production and material essential for life for herdsmen in the Tibetan pasture (Gu et al. 2007). The yak industry was listed as the first batch of "Promote the Autonomous Prefecture and Enrich the People" key projects in Seda County, Ganzi Tibetan autonomous prefecture, Sichuan Province, China.

Although yaks have a strong ability to resist disease, diarrhea, usually caused by Shiga toxin-producing *Escherichia coli* remains a major health problem (Bandyopadhyay et al. 2009; Bai et al. 2013). According to an earlier investigation, the main antimicrobial agents used in the treatment of yak for diarrhea were sulfamethoxazole, sulphafurazole, florfenicol, penicillin, amoxicillin, and streptomycin (Y.Y. unpubl. data). Although sulfonamides have been banned from use in food-producing animals for many years, sulfamethoxazole and sulphafurazole are still used in the Tibetan pasture because of a shortage of medical services and supplies. In addition, sulfonamide, norfloxacin, and amoxicillin are used in herdsmen for bacterial disease.

Resistance to sulfonamides in *E. coli* can result from the acquisition of an alternative deoxyhypusine synthase gene (*sul*) (Radstrom and Swedberg 1988), whose product has a lower affinity for sulfonamides. Three sulfonamide resistance genes (*sul1, sul2,* and *sul3*) in gram-negative bacteria have been described (Perreten and Boerlin 2003)

TABLE 1. Origins of the *Escherichia coli* isolates from Yaks (*Bos grunniens*) and herdsman in Seda County, Ganzi Tibetan autonomous prefecture, Sichuan Province, China, 2009–2011.

Farm	Antimicrobials used in herdsmen	Antimicrobials used in yaks	No. of samples (yak/herdsman)	No. of *E. coli* isolates (yak/herdsman)
Wuqing pasture	Sulfonamide, norfloxacin, amoxicillin	Sulfonamide, streptomycin, penicillin, florfenicol	20/10	14/8
Gaoshan pasture	Sulfonamide, norfloxacin, amoxicillin	Sulfonamide, streptomycin, penicillin, florfenicol	20/10	12/6
Qingjia pasture	Sulfonamide, norfloxacin, amoxicillin	Sulfonamide, streptomycin, penicillin, florfenicol	20/10	11/7
Aga pasture	Sulfonamide, amoxicillin	Sulfonamide, streptomycin, penicillin, florfenicol	25/10	15/9
Kangle pasture	Unknown	Unknown	25/10	12/9
Daze pasture	Sulfonamide	Sulfonamide	25/10	10/7
Niannong pasture	Sulfonamide, norfloxacin, amoxicillin	Sulfonamide, penicillin	25/10	13/9
Xiangyang pasture	Sulfonamide	Sulfonamide, streptomycin, penicillin	25/10	12/7
Dazhang pasture	Sulfonamide, norfloxacin, amoxicillin	Sulfonamide, streptomycin, penicillin	25/10	15/8
Total			210/90	114/70

and have been frequently detected in *E. coli* from origins around the world (Enne et al. 2001; Wu et al. 2010). Florfenicol is a synthetic, broad-spectrum, fluorinated derivative of chloramphenicol approved exclusively for veterinary use (Schwarz et al. 2004). Six florfenicol resistance genes have been detected in a wide variety of bacteria including *floR* and *cmlA* in several gram-negative bacteria (Schwarz et al. 2004): *cfr* and *fexA* in different *Staphylococcus* spp. (Kehrenberg and Schwarz 2004) and *pexA* and *estDL136* in uncultured bacteria (Lang et al. 2010; Tao et al. 2012).

Little information is available regarding the epidemiology of antimicrobial-resistant *E. coli* isolated from yaks and herdsmen in the Tibetan pasture. In this study we address two questions: 1) What are the resistance phenotypes and genotypes of *E. coli* isolated from yaks and herdsmen? 2) Are drug-resistant bacteria and resistance genes shared by yaks and herdsmen?

MATERIALS AND METHODS

Sample population

We collected samples from Seda County, Ganzi Tibetan autonomous prefecture, Sichuan Province, China, which is on the southeastern margin of the Qinghai-Tibet Plateau at an average elevation of 3,893 m. We collected 300 nonduplicated fecal samples of yaks ($n=210$) and herdsmen ($n=90$) from nine Tibetan pastures between May 2009 and October 2011 (Table 1). We followed yaks and collected specimens immediately after fecal voiding. Fresh fecal samples were placed into 2 mL sterile tubes containing 80% glycerin and 20% skim milk, stored on ice packs, and transported to the laboratory for isolation of *E. coli*. This study was approved by the local ethics board of the Medical Ethics Committee of Sichuan University, and consent was obtained from all herdsmen in nine Tibetan pastures.

Bacterial isolates

The samples were inoculated into tryptone soya broth and incubated at 37 C for 18 h with shaking at 200 rpm. All of the isolates were presumptively identified by phenotypic methods, including colony morphology on

TABLE 2. PCR primers used in this study.

Gene	Description	Sequence (5′–3′)	Amplicon size (base pairs)	Reference
sul1	Dihydropteroate synthetase	F: CGGCGTGGGCTACCTGAACG R: GCCGATCGCGTGAAGTTCCG	433	Kerrn et al. 2002
sul2	Dihydropteroate synthetase	F: GCGCTCAAGGCAGATGGCATT R: GCGTTTGATACCGGCACCCGT	293	Kerrn et al. 2002
sul3	Dihydropteroate synthetase	F: AGATGTGATTGATTTGGGAGC R: TAGTTGTTTCTGGATTAGAGCCT	443	Zhang et al. 2009
cfr	rRNA methyl-transferase	F: TGAAGTATAAAGCAGGTTGGGA GTCA R: ACCATATAATTGACCACAAGCA GC	746	Wang et al. 2012
flor	Efflux protein	F: GGCTTTCGTCATTGCGTCTC R: ATCGGTAGGATGAAGGTGAGGA	650	Zhang et al. 2009
fexA	Efflux protein	F: GTACTTGTAGGTGCAATTACGG CTGA R: CGCATCTGAGTAGGACATAGC GTC	1,272	Kehrenberg and Schwarz 2004
cmlA	Efflux protein	F: TGCCAGCAGTGCCGTTTAT R: CACCGCCCAAGCAGAAGTA	900	Zhang et al. 2009
pexA	Efflux protein	F: TTCACTGCAGGGATCGTGAC R: CAACTGCAGAAAAGCGAAAAG	1,701	Lang et al. 2010
estDL 136	Hydrolytic enzyme	F: TGCCCGCACCCGATTTCT R: GATTGGATGCACCTCGTTCTA	864	Tao et al. 2012

MacConkey agar and eosin-methylene blue agar, and gram stain. Identification was later confirmed by the Vitek system (bioMérieux, Durham, North Carolina, USA). The isolates were stored in Luria-Bertani broth containing 15% glycerol at −80 C until analysis. The 'O' serogroup of the *E. coli* isolates was determined using a slide agglutination test. The antiserum used for O serotyping was produced by Tianjin Biochip Corporation (Tianjin, China).

Antimicrobial susceptibility testing

The susceptibilities of the isolates to 15 antimicrobials were determined using the standard Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI 2010) and performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals (CLSI 2008). The following antimicrobial disks from Oxoid Ltd. (Basingstoke, UK) were used: ampicillin, amoxicillin, ceftiofur, gentamicin, neomycin, streptomycin, doxycycline, tetracycline, spectinomycin, nalidixic acid, norfloxacin, ciprofloxacin, sulphafurazole, sulfamethoxazole, and florfenicol. The internationally recognized control strain for antimicrobial susceptibility testing, *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, Virginia, USA), was used as the quality control strain.

PCR for detecting sulfonamide and florfenicol resistance genes

Escherichia coli isolates, which had resistance to sulfonamide, were amplified (sulfonamide-resistant genes *sul1*, *sul2*, and *sul3*) by PCR using previously described primers (Kerrn et al. 2002; Zhang et al. 2009). The rRNA methylase gene *cfr*; four florfenicol exporter genes *floR, fexA, cmlA,* and *pexA*; and the newly discovered florfenicol hydrolytic enzyme gene *estDL136,* were amplified in florfenicol-resistant isolates (Kehrenberg and Schwarz 2004; Zhang et al. 2009; Lang et al. 2010; Tao et al. 2012). The PCR primers are described in Table 2. The PCR products were purified using a QIA quick PCR Purification Kit (QIAGEN Inc., Valencia, California, USA), cloned to a pMD18-T vector, and transformed into JM109-competent recipient *E. coli*. Positive clones were sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China) on both strands by automated sequencing with an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). The resulting DNA sequence data was

TABLE 3. Antimicrobial susceptibility of *E. coli* isolates from yaks (*Bos grunniens*) and herdsmen, Seda County, Ganzi Tibetan autonomous prefecture, Sichuan Province, China, 2009–2011.

Antimicrobial	Herdsmen (*n*/total)		Yaks (*n*/total)		*P* value for nonsusceptibility
	% Resistant	% Intermediate	% Resistant	% Intermediate	
Sulfamethoxazole[a]	57 (40/70)	0 (0/70)	44.0 (50/114)	0.0 (0/114)	0.08
Sulphafurazole[a]	51 (36/70)	3 (2/70)	40.4 (46/114)	0.0 (0/114)	0.09
Florfenicol[b]	4 (3/70)	3 (2/70)	11.4 (13/114)	1.8 (2/114)	0.18
Streptomycin[a]	20 (14/70)	9 (6/70)	1.8 (2/114)	3.5 (4/114)	<0.001
Spectinomycin[b]	14 (10/70)	6 (4/70)	0.0 (0/114)	0.0 (0/114)	<0.001
Ampicillin[a]	23 (16/70)	17 (12/70)	6.1 (7/114)	9.6 (11/114)	<0.001
Amoxicillin[a]	26 (18/70)	20 (14/70)	8.8 (10/114)	3.5 (4/114)	<0.001
Ceftiofur[b]	11 (8/70)	20 (14/70)	0.0 (0/114)	0.0 (0/114)	<0.001
Neomycin[b]	0 (0/70)	3 (2/70)	0.0 (0/114)	0.9 (1/114)	0.16
Gentamicin[a]	11 (8/70)	6 (4/70)	3.5 (4/114)	1.8 (2/114)	0.02
Doxycycline[a]	6 (4/70)	11 (8/70)	0.0 (0/114)	3.5 (4/114)	0.006
Tetracycline[a]	11 (8/70)	14 (10/70)	0.0 (0/114)	2.6 (3/114)	<0.001
Nalidixic acid[a]	11 (8/70)	0 (0/70)	0.0 (0/114)	0.0 (0/114)	0.004
Norfloxacin[a]	9 (6/70)	3 (2/70)	0.0 (0/114)	0.9 (1/114)	0.009
Ciprofloxacin[a]	3 (2/70)	20 (14/70)	0.0 (0/114)	0.9 (1/114)	<0.001

[a] CLSI 2010.

[b] CLSI 2008.

compared with data from GenBank using the Basic Local Alignment Search Tool (National Center for Biotechnology Information 2015).

Pulsed-field gel electrophoresis (PFGE)

We performed PFGE for 13 *floR*-positive isolates from herdsmen and yaks to assess overlap in the two neighboring pastures following the criteria of Tenover et al. (1995). Three florfenicol-resistant isolates from herdsmen were included. Electrophoresis was performed using a CHEF DRIII System (Bio-Rad Laboratories Inc., Hercules, California, USA) after *Xba I* digestion. The PFGE conditions were: voltage, 6 V/cm; included angle, 120°; pulse time, 2.70~29.00 s; electrophoresis time, 20 h; and temperature, 14 C. Software Quantity One version 4.62 (Bio-Rad Laboratories) was used to analyze genetic relationship among isolates. Similarity between fingerprints was calculated with the Dice coefficient (Vali et al. 2014). A cluster analysis was performed using the unweighted pair-group method with average linkages.

Statistical analysis

Fisher's exact test and a χ^2 tests were used to compare the susceptibility testing results of the herdsmen and yak isolates. A $P<0.05$ value was considered statistically significant. The association of *E. coli* serotypes and antibiotic resistance was tested by the Pearson's correlation test using SPSS.19 software (IBM Corporation, Armonk, New York, USA).

RESULTS

Escherichia coli isolates and 'O' serogroup

We isolated 184 nonrepetitive isolates of *E. coli* (yaks, $n=114$; herdsmen, $n=70$) (Table 1). The isolates from yaks were divided into 24 'O' serogroups, and the dominant types included O101 (12.3%), O2 (9.6%), O68 (7.0%), O132 (7.0%), O78 (5.3%), O171 (5.3%), O22 (4.4%), O142 (4.4%), and O165 (4.4%). The strains originating from herdsmen were divided into 12 'O' serogroups, and the dominant types included O81 (14%), O30 (11%), O5 (11%), O165 (9%), O132 (9%), and O101 (9%).

Antimicrobial susceptibility testing

The antimicrobial resistance of these isolates is shown in Table 3. The strains isolated from yaks showed the highest resistance rate to sulfamethoxazole (44%), followed by sulphafurazole (40.4%), florfenicol (11.4%), amoxicillin (8.9%), ampicillin (6.1%), gentamicin (3.5%), and streptomycin

TABLE 4. Prevalence of resistance genes in sulfonamides and florfenicol resistance *E. coli* isolates in yaks (*Bos grunniens*) and herdsmen, Seda County, Ganzi Tibetan autonomous prefecture, Sichuan Province, China, 2009–2011.

Resistance gene	Source, *n* (%)	
	Herdsmen	Yaks
	n=40	*n*=50
sul genes	37 (93)	47 (94)
sul 1	17 (43)	18 (36)
sul 2	11 (28)	26 (52)
sul 3	3 (8)	0 (0)
sul 1 + sul 2	5 (13)	3 (6)
sul 1 + sul 3	1 (3)	0 (0)
	n=16	*n*=3
flor	14 (88)	3 (100)
cmlA	3 (19)	1 (33)
cfr	0 (0)	0 (0)
fexA	0 (0)	0 (0)
PexA	0 (0)	0 (0)
est136DL	0 (0)	0 (0)

(1.8%). All isolates were sensitive to neomycin, doxycycline, tetracycline, norfloxacin, ciprofloxacin, nalidixic acid, spectinomycin, and ceftiofur. The resistance ratio of isolates from herdsmen was 57% and 51% to sulfamethoxazole and sulphafurazole, followed by amoxicillin (26%), ampicillin (23%), streptomycin (20%), spectinomycin (14%), tetracycline (11%), ceftiofur (11%), norfloxacin (9%), doxycycline (6%), florfenicol (4%), ciprofloxacin (3%), and neomycin (0%). Highly significant differences in resistance to streptomycin, spectinomycin, ampicillin, amoxicillin, ceftiofur, ciprofloxacin, and tetracycline were observed between isolates from herdsmen and yaks ($P<0.001$).

Prevalence of resistance genes

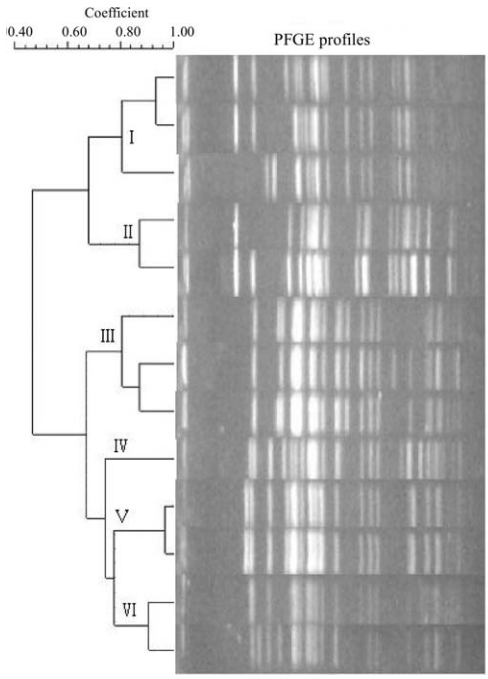
Among the 90 sulfonamide-resistant isolates, *sul* genes were present in 93% (84/90), with *sul1*, *sul2,* and *sul3* detected alone or in combination in 44 (49%), 45 (50%), and 4 (4%) of the strains, respectively. No strain was positive for *sul2 + sul3* and *sul1 + sul2 + sul3* in combination. Fourteen (87%) *floR* genes were detected in 16 florfenicol-resistant isolates from yaks and three *floR* genes were detected in three florfenicol-resistant isolates from herdsmen. Four *cmlA* genes were found in 19 florfenicol-resistant isolates. In addition, *cfr, fexA, pexA,* and *estDL136* genes were not detected in any of the florfenicol-resistant isolates. All of the positive clones were sequenced. The basic local alignment search tool (BLAST) results showed that all of the sequences obtained in this study were highly homologous with sequences in GenBank (≥99%). Detailed information on these resistance genes are provided in Table 4.

Pulsed-field gel electrophoresis (PFGE) and genetic relatedness analysis of strains

Six different *Xba I*-PFGE patterns (I~VI) of *floR*-positive isolates were observed. Some isolates had similar patterns. In pattern I, WY8 isolated from yaks was similar to WH4 and WH7 isolated from herdsmen in Wuqin pasture (similarity coefficient 0.83). There was a similarity coefficient of 0.86 between WY12 isolated from yaks in Wuqin pasture and GY13 from yaks in Gaoshan pasture, which had similar PFGE patterns to GH12 isolated from herdsman in Gaoshan pasture (Fig. 1). No significant association was found between serotypes and resistance phenotypes.

DISCUSSION

We found 24 'O' serogroups in *E. coli* isolates from yaks, which was significantly different from *E. coli* isolated from captive yaks in India (Bandyopadhyay et al. 2009). Only three serogroups (O2, O22, and O78) reported in this study were also observed in Shiga toxin-producing *E. coli* in yaks in the Qinghai-Tibetan Plateau (Bai et al. 2013). This implies that diverse 'O' serogroups of *E. coli* were present in yaks in different regions. Both O101 and O132 were simultaneously present in yaks and herdsmen, suggesting overlap of 'O'



Strain	Isolate region	Source	Serogroup	Antibiotic resistance profiles
WH4	Wuqin pasture	herdsman	O5	SMZ, SF, FFC, AMP, AML, NA
WH7	Wuqin pasture	herdsman	O5	SMZ, SF, FFC, AMP, AML, NA
WY8	Wuqin pasture	yak	O5	SMZ, SF, FFC
WY17	Wuqin pasture	yak	O68	FFC, AMP, AML
WY18	Wuqin pasture	yak	O68	SMZ, SF, FFC
GH12	Gaoshan pasture	herdsman	O101	SMZ, SF, FFC
WY12	Wuqin pasture	yak	O101	SMZ, SF, FFC
GY13	Gaoshan pasture	yak	O101	SMZ, SF, FFC
GY3	Gaoshan pasture	yak	O17	SMZ, SF, FFC
GY14	Gaoshan pasture	yak	O22	SMZ, SF, FFC
GY16	Gaoshan pasture	yak	O22	SMZ, SF, FFC, STR
GY19	Gaoshan pasture	yak	O15	SMZ, SF, FFC
GY22	Gaoshan pasture	yak	O15	SMZ, SF, FFC

FIGURE 1. Pulsed-field gel electrophoresis (PFGE) patterns and genetic relatedness analysis of strains of *floR*-positive *Escherichia coli* isolated after digestion of total DNA with *Xba I*, from yaks (*Bos grunniens*) and herdsman from pastures in Seda County, Ganzi Tibetan autonomous prefecture, Sichuan Province, China, 2009–2011. SMZ = sulfamethoxazole; SF = sulphafurazole; AMP = ampicillin; AML = amoxicillin; FFC = florfenicol; NA = nalidixic acid; STR = streptomycin.

serogroups in *E. coli* isolates from humans and animals.

Escherichia coli in our study had a higher resistance to sulfanilamide than to other antimicrobial agents but much lower than the strains isolated from other commercial animals in China (Lei et al. 2010; Tian et al. 2012). The reason for this low rate of antibiotic resistance might be the short-term use of antibiotics in Tibetan pastures. The antibiotic-resistant phenotypes of isolates from herdsmen were more complicated than isolates from yaks (Table 3). According to the survey of antibiotic use in Tibetan pastures (Table 1), sulfanilamides (sulphafurazole and sulfamethoxazole) were mostly used to treat yaks with diarrhea. However, herdsmen usually used sulphafurazole, sulfamethoxazole, ampicillin, and amoxicillin for their own illness. Our results were in accordance with the custom of using these antimicrobial agents in pastures. Although florfenicol is prohibited for use in humans, florfenicol resistance was detected in three isolates from herdsmen. This indicates that antibiotic resistant isolates or genes might be transmitted from yaks to herdsmen.

We mainly amplified the sulfonamide resistance genes in *E. coli* due to the high resistance to sulfanilamide. The florfenicol resistance gene was also included because of the florfenicol-resistant *E. coli* found in herdsmen. Among 90 sulfanilamide-resistant strains, the positive rate of *sul* genes was 93%, which revealed that the *sul* genes were most prevalent in the Tibetan pasture. A nearly perfect correlation between genotype and phenotype was found with regard to sulfanilamide. The *sul1* and *sul2* genes were common in sulfanilamide-resistant strains, results which differ from other research on swine

and chickens (Kozak et al. 2009). Seventeen florfenicol resistance genes (*floR*) were found in 19 florfenicol-resistant *E. coli*, indicating that efflux of the drug is the main resistance mechanism to florfenicol (Blickwede and Schwarz 2004). The *cfr*, *fexA*, *pexA*, and *estDL136* genes were not amplified in any of the florfenicol-resistant isolates in our study. A possible reason is that the *cfr* and *fexA* genes were mainly found in gram-positive bacteria, whereas only one *cfr* gene-positive strain has been found in *E. coli* (Wang et al. 2012). The *pexA* and *estDL136* genes were only detected in uncultured bacteria from soil (Lang et al. 2010; Tao et al. 2012). However, to the best of our knowledge, this is the first report of the *floR* gene in *E. coli* isolated from yaks and herdsmen.

A few strains of *E. coli* isolated from yaks and herdsmen in different pastures had the same serogroups, antimicrobial-resistant gene, and PFGE patterns, suggesting that the potential risk of antimicrobial-resistant *E. coli* would disseminate in yaks and herdsmen through the spread of clones. This result might be caused by lifestyle and poor sanitary conditions in Tibetan pastures. The Tibetan people have a close relationship with yaks through drinking yak milk, eating air-dried yak meat, and burning yak droppings for energy. This could imply that, although the drug is used strictly in animals, the use of florfenicol might select for and amplify resistance to antimicrobials that are relevant to human health (O'Brien 2002). Previous research has shown that antimicrobial-resistant bacteria could spread from animals to humans (Winokur et al. 2001; Rwego et al. 2008), which was proven by our research. Continuous surveillance of antibiotic resistance should be performed in Tibetan pastures.

In conclusion, we report the *floR* gene in *E. coli* isolated from yaks and herdsmen and a high presence of sulfonamide resistance genes in *E. coli* isolated from the Tibetan pastures. We also demonstrated that the antibiotic-resistant *E. coli* and resistance genes (such as *floR*) might directly disseminate between humans and yaks due to the lifestyle habits (burning yak droppings for energy, infrequent hand and body washing, and eating air-dried beef) of herdsmen in the Tibetan pastures; more attention should be paid to these aspects to reduce dissemination.

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