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Antibiotic resistance and screening of the resistant genes of *Escherichia coli* (*E. coli*) isolated from diarrheal yak calves in Sichuan Province, China

Li, K.[1,§], Wang, X.Q.[1,2,§], Shahzad, M.[3], Zhang, H.[1], Zhao, X.D.[4], Jiang, X.[1], Mehmood, K.[1,3], Han, Z.Q.[1,5], Wang, L.[1] and Li, J.K.[1,6,*]

[1]College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, People's Republic of China

[2]DeQing Animal Husbandary and Veterinary Bureau, Huzhou, 313299, People's Republic of China

[3]University College of Veterinary & Animal Sciences, The Islamia University of Bahawalpur, 63100 Pakistan

[4]Longri original breeding farm of Sichuan province, People's Republic of China

[5]College of Agriculture and Forestry Science, Linyi University, Linyi 276005, People's Republic of China

[6]Laboratory of Detection and Monitoring of Highland Animal Disease, Tibet Agriculture and Animal Husbandry College, Linzhi 860000 Tibet, People's Republic of China

[*]Corresponding author e-mail: lijk210@sina.com

[§]These two authors contributed equally in this study.

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Abstract. This study was conducted to determine the antibiotic and screening resistance genes of *Escherichia coli* (*E. coli*) isolated from diarrheal yak calves from high remote plateau in Sichuan, China. A total 41 rectal swabs were obtained from diarrheal yak calves. *E. coli* were isolated and identified. The antimicrobial sensitivity was tested by piloting the disk diffusion method for 21 antibiotics. Polymerase chain reaction was employed to detect the resistance genes. The results showed that the drug resistance ranged from 2.4% (amikacin) to 53.7% (tetracycline), while no isolates were found resistant to neomycin and polymyxin B. Multi-drug resistance was detected in 4.9% isolates to 17 antimicrobial agents; and 24.4% isolates were found susceptible to all antimicrobial agents. The aminoglycoside resistance genes of aac(3)-lla, ant(3')-la and aph(3')-lla was positive in 4.9%, 2.1% and 7.3% *E. coli* isolates respectively. The 4.9% and 2.1% of *E. coli* isolates were detected in b-Lactam resistance genes of TEM and CTX-M, respectively; and 12.2% and 4.9% of *E. coli* isolates were found to have Tetracycline resistance genes of tetM and tetA, respectively. The present study reveals that the yak calves from high cold plateau are potential reservoir of *E. coli* with widely distributed multiple drug resistance which requires the attention of concerned authorities regarding the use of non-standard antibiotics.

INTRODUCTION

Escherichia coli (*E. coli*), a member of Enterobacteriaceae family is a gram negative opportunistic commensal bacterium that inhabits the guts of most vertebrates (Kaper *et al.*, 2004; Massot *et al.*, 2017; Yumi *et al.*, 2017). Cattle and other ruminants are the most important animal reservoir for *E. coli* (Stein and Katz, 2017). Multiple food and water-borne outbreaks caused by *E. coli* worldwide have made this bacterium a serious threat for public health (Suardana *et al.*, 2017).

Antibiotic agents have been extensively utilized to prevent and treat infectious diseases in veterinary and human medicine (Xuan Nguyen *et al.*, 2017). However, antimicrobial resistance, especially multidrug-resistance has become a serious issue to public health due to overuse or abuse of antibiotics (Zou *et al.*, 2011; Guo *et al.*, 2015; Xuan Nguyen *et al.*, 2017). Animals are commonly considered as important reservoir for antimicrobial-resistant *E. coli* (Guo *et al.*, 2015).

The long haired yak (*Bos mutus*), a species of bovine family, are mainly found on the Himalayan region of the South Central Asia including China, India, Russia, Mongolia, Bhutan, Nepal and other countries (Li *et al.*, 2014; Li *et al.*, 2016). In China, yaks are found in four western provinces (Qinghai, Tibet, Sichuan and Gansu) with a population of 14 million accounting for approximately 90.0% of the yaks in the world (Han *et al.*, 2013; Li *et al.*, 2017). Out of these, 4 million yaks live on the high cold plateau (4500 m) in Sichuan province of China. The great economic value of yak milk, meat, dung, and wool makes this animal an important species for the native herdsmen (Li *et al.*, 2014; Li *et al.*, 2015).

Diarrhea is a serious disease that affects the fertility, weight gain and milk production of cattle leading to significant economic losses (Han *et al.*, 2017). Calves particularly, the perinatal ones are seriously affected by this disease resulting in death (Tsuchiaka *et al.*, 2016). Previous studies have reported various bacterial pathogens causing diarrhea in cattle with *E. coli K99*, *Salmonella* species and *Mycobacterium avium* subspecies paratuberculosis as prevalent infectious agents (de Graaf *et al.*, 1999; Chi *et al.*, 2002; Bartels *et al.*, 2010). To date, scarce knowledge is available about the antibiotic sensitivity and serotypes of *E. coli* infection in diarrheal yak-calves in Sichuan province of China. Therefore, this study was designed to test for antibiotic resistance and screening of the resistant genes of *E. coli* isolated from diarrheal yak-calves in Sichuan, China.

MATERIALS AND METHODS

Sample collection, isolation, and identification

A total of 41 rectal swabs were obtained from diarrheal yak-calves in Hongyuan (average altitude 4300 m; annual average temperature 1.4°C) of Sichuan, China during 2016 (Fig. 1). Collections were subsequently transported to the clinic laboratory of Huazhong Agricultural University, Wuhan for further tests.

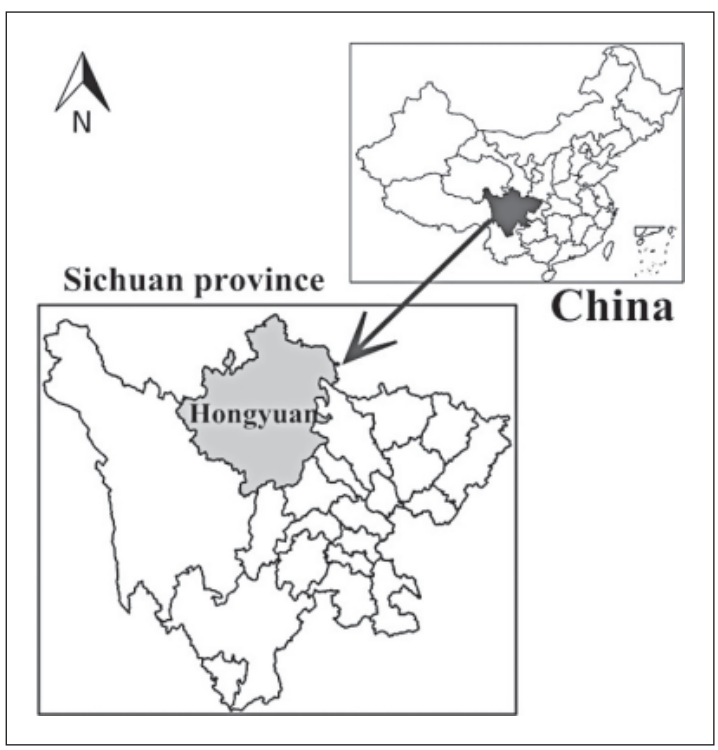

Figure 1. The geographical site for sample collection.

All isolates were enriched by employing commercial nutrient broth and streaking on MacConkey agar (GE Hangwei Medical Systems Co., Ltd., Beijing, China). The pink-colored colonies were then selected and inoculated on EMB (eosin methylene blue agar), that presented as characteristic of greenish metallic-colored colonies on *EMB* and were recognized as *E. coli*. Biochemical analysis was confirmed for *E. coli* strains through the API 20E system (BioMerieux, Marcy-l Etoile, France). The identified strains were suspended in TSB (Tryptic Soya Broth) and stored at -80°C in 20.0% glycerol for further experiments.

Antibiotic sensitivity detection

We piloted the disk diffusion method to test the antimicrobial sensitivity profile of *E. coli* isolate, with the instruction of the criteria described by the Clinical and Laboratory Standards Institute (CLSI, 2014). Mueller-Hinton agar was utilized with each of the following 21 commercial antimicrobial agents (Hangzhou Microbial Reagent Co., Ltd., Hangzhou, China and Hangzhou Binhe Microorganism Reagent Co., Ltd., Hangzhou, China): neomycin (Neo, 30µg) (Catalog # C109), kanamycin (Kan, 30µg) (Catalog # C015), gentamicin (Gen, 10µg) (Catalog # C017), amikacin (An, 30µg) (Catalog # C016), furazolidone (Fur, 300µg) (Catalog # C029), trimethoprim/sulfamethoxazole (SMZ-TMP, 23.75/1.25µg) (Catalog # C027), polymyxin B (PB, 300µg) (Catalog # C025), norfloxacin (Nor, 10µg) (Catalog # C033), ofloxacin (Ofl, 5µg) (Catalog # C044), ciprofloxacin (Cip, 5µg) (Catalog # C045), chloramphenicol (Chi, 30µg) (Catalog # S1063), carbenicillin (Cb, 100µg) (Catalog # S1004), ampicillin (Amp, 10µg) (Catalog # S1001), piperacillin (Prl, 100µg) (Catalog # S1008), doxycycline (Dox, 30µg) (Catalog # S1037), tetracycline (TET, 30µg) (Catalog # S1036), cephazolin (Cfz, 30µg) (Catalog # S1012), cefalexin (Cl, 30µg) (Catalog # S1011), ceftriaxone (Cro, 30µg) (Catalog # S1020), cefoperazone (Cfp, 30µg) (Catalog # S1021), and cefradine (Ce, 30µg) (Catalog # S1013). Each of the detections was performed in triplicate. Laboratory stored *E. coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 700603) were applied as positive and negative control strains.

DNA extraction and resistance genes amplification

Total DNA extraction of *E. coli* strains were carried out by boiling as described by Levesque *et al.* (1995). Resistance genes were amplified by employing primers and methodology as described in Table 1. All amplifications were detected by 1.5% agarose gel electrophoresis.

Statistical analysis

Variables were expressed as percentages (%). The significant association between antibiotic-resistant sensitivity was determined using the Pearson's Chi-squared test by utilizing the IBM SPSS Statistics 20.0 (SPSS Somers, NY). P values <0.05 were considered significant.

RESULTS

Phenotypic testing of antimicrobial resistance

Part of the antimicrobial susceptibility results are shown in Fig. 2. The topmost resistance rates were detected against tetracycline (53.7%), cefradine (51.2%), doxycycline (48.8%), carbenicillin (46.3%), ampicillin (46.3%), ciprofloxacin (41.5%) and norfloxacin (41.5%); and moderate rates of resistance was observed against Trimethoprim/sulfamethoxazole (36.6%), ofloxacin (26.8%), piperacillin (22.0%) and cefalexin (22.0%). However, low antimicrobial resistance of 14.6%, 14.6%, 12.2%, 9.8%, 7.3%, 7.3%, 7.3% and 2.4% was found against gentamicin, chloramphenicol, kanamycin, cefoperazone, furazolidone, cephazolin, ceftriaxone, and amikacin, respectively. No isolates were found to be resistant to neomycin and polymyxin B (Fig. 3). 4.9% of the isolates were found to be resistant to multi-drug for 17 antimicrobial agents, however, 24.4% isolates were found to be susceptible to all the antimicrobial agents (Fig. 4) (Table 2).

Table 1. Nucleotide sequences of PCR primer sets utilized in this study

Resistance gene	Primer sequence (5'–3')	Length (bp)	Reference
ant(3')-la	ATCTGGCTATCTTGCTGACA TATGACGGGCTGATACTGG	284	Zhang *et al.* (2009)
aph(3')-lla	TGACTGGGCACAACAGACAA CGGCGATACCGTAAAGCAC	677	Guo *et al.* (2015)
aac(3)-lla	ACCCTACGAGGAGACTCTGAATG CCAAGCATCGGCATCTCATA	384	Zhang *et al.* (2009)
aac(6')-lb	ATGACCTTGCGATGCTCTATG CGAATGCCTGGCGTGTTT	486	Zhang *et al.* (2009)
blaCTX-M	TTTGCGATGTGCAGTACCAGTAA CGATATCGTTGGTGGTGCCATA	544	Edelstein *et al.* (2003)
blaTEM	ATGAGTATTCAACATTTCCGTG TTACCAATGCTTAATCAGTGAG	840	Guo *et al.* (2015)
blaSHV	TGGTTATGCGTTATATTCGCC GCTTAGCGTTGCCAGTGCT	1051	Guo *et al.* (2015)
tetA	GGCACCGAATGCGTATGAT AAGCGAGCGGGTTGAGAG	480	Guo *et al.* (2015)
tetM	CTGGGCTGCTTCCTAATGC AGCTGTCCCTGATGGTCGT	580	Guo *et al.* (2015)
tetC	CTCAGTATTCCAAGCCTTTC CTAAGCACTTGTCTCCTGTT	416	Guo *et al.* (2015)

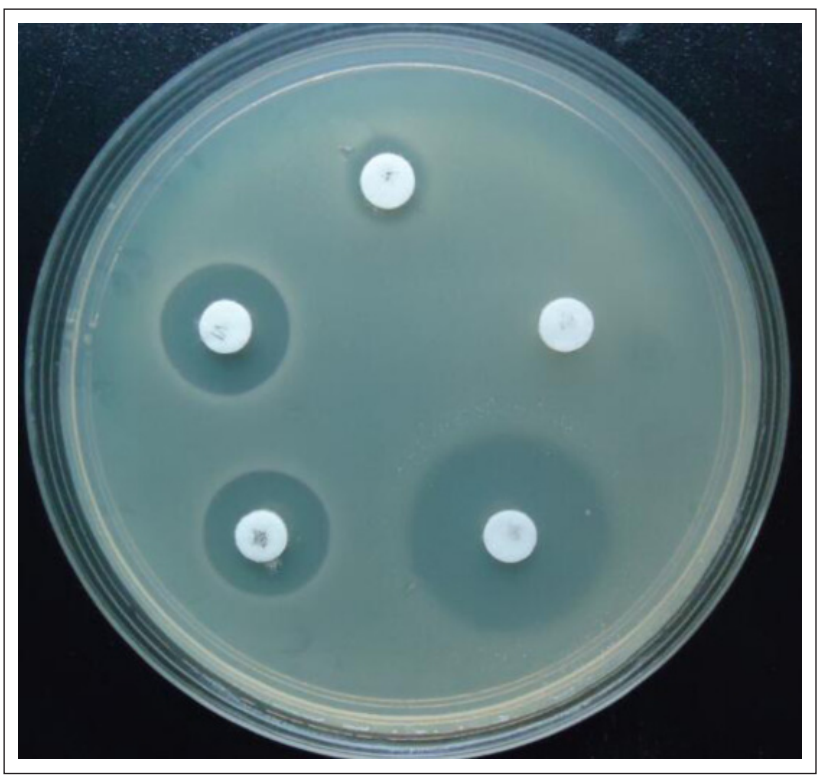

Figure 2. The antimicrobial susceptibility test by disc diffusion method.

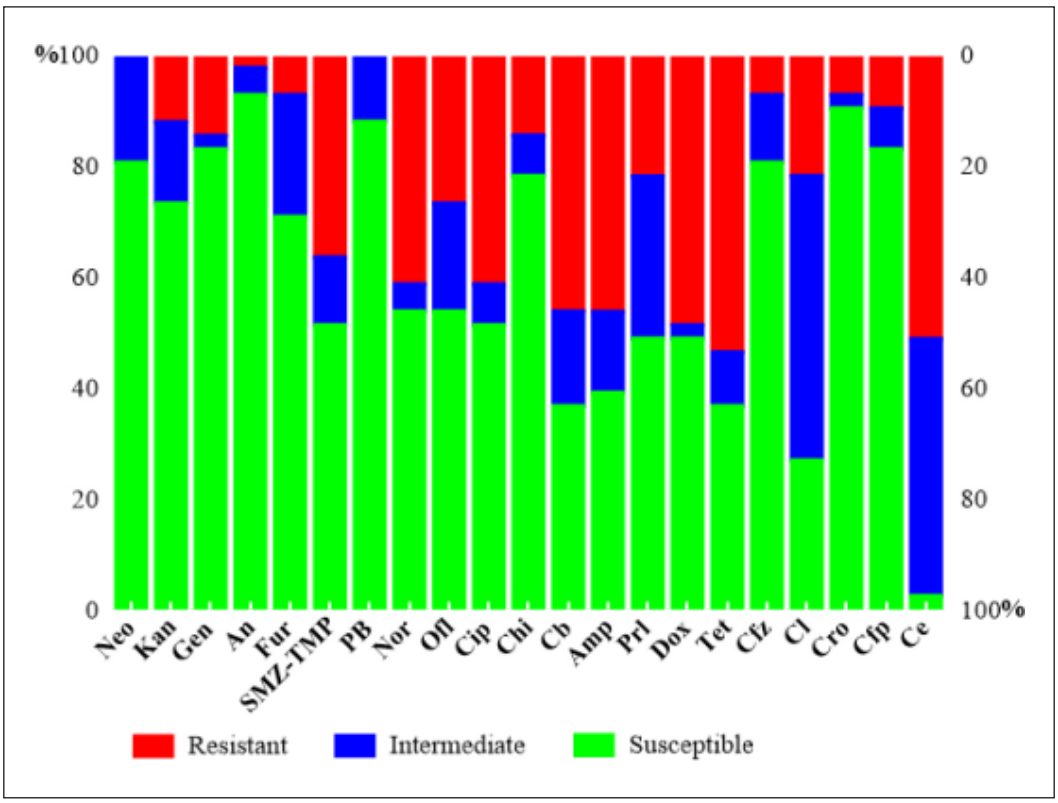

Figure 3. The results of antimicrobial susceptibility of the *E. coli* strains isolated from yak calves.

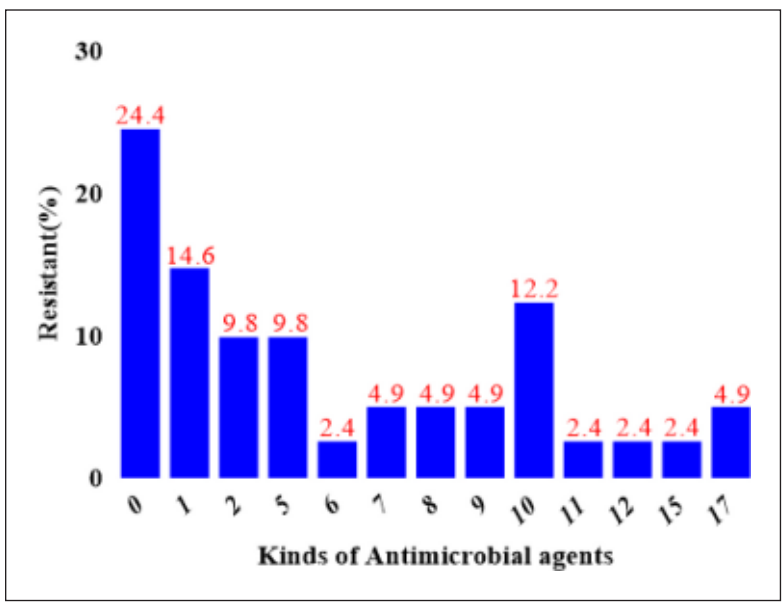

Figure 4. The results of multi-drug resistance of the *E. coli* strains isolated from yak calves.

Table 2. The detail multidrug resistance of *E. coli* strains isolated from yak calves

No.	Antimicrobial agent
1	Ce
2	Ce/Cb, Dox/Tet, Cl/Ce, Ofl/Cip
5	Dox/Tet/Amp/Prl/Gen, Dox/Tet/Amp/Cb/Prl, Dox/Tet/Nor/Ofl/Cip, Dox/Tet/Nor/Cip/SMZ-TMP
6	Dox/Tet/Amp/Cb/Prl/Ce
7	Dox/Tet/SMZ-TMP/Cb/Amp/Chi/Nor, Dox/Tet/SMZ-TMP/Cb/Amp/Chi/Kan
8	Dox/Tet/ Cb/Amp/Nor/Cip/Ofl/SMZ-TMP, Dox/Tet/Cb/ Amp/Nor/Cip/Ce/Prl
9	Dox/Tet/Cb/Amp/Nor/Ofl/Cip/Gen/SMZ-TMP, Dox/Tet/Cb/Amp/Nor/Ofl/Cip/Gen/Ce/
10	Dox/Tet/Cb/Amp/Nor/Cip/SMZ-TMP/Ce/Cl/Chi, Dox/Tet/Cb/Amp/Nor/Cip/SMZ-TMP/Ce/Cl/Cfp Dox/Tet/Cb/Amp/Nor/Cip/SMZ-TMP/Ce/Cl/Prl, Dox/Tet/Cb/Amp/Nor/Cip/SMZ-TMP/Ce/Cl/Chi Dox/Tet/Cb/Amp/Nor/Cip/SMZ-TMP/Ce/Ofl/Gen
11	Dox/Tet/Cb/Amp/Nor/Cip/SMZ-TMP/Ce/Cl/Ofl/Prl
12	Tet/Cb/Amp/Nor/Cip/SMZ-TMP/Ce/Ofl/Kan/Gen/An/Fur
15	Dox/Tet/Cb/Amp/Nor/Cip/SMZ-TMP/Ce/Cl/Ofl/Prl/Cfz/Cfp/Kan/Cro
17	Dox/Tet/Cb/Amp/Nor/Cip/SMZ-TMP/Ce/Cl/Ofl/Prl/Cfz/Cfp/Kan/Cro/Chi/Fur Gen/Tet/Cb/Amp/Nor/Cip/SMZ-TMP/Ce/Cl/Ofl/Prl/Cfz/Cfp/Kan/Cro/Chi/Fur

PCR amplification of antimicrobial resistance genes

In this study, some genes were amplified successfully (Fig. 5); 4.9%, 2.1% and 7.3% *E. coli* isolates were tested out for aminoglycoside resistant genes of *aac(3)-lla*, *ant(3')-la* and *aph(3')-lla* respectively. Only 4.9% and 2.1% of *E. coli* isolates were detected for b-Lactam resistance genes of *TEM* and *CTX-M* respectively while 12.2% and 4.9% of *E. coli* isolates were observed for Tetracycline resistance genes of *tetM* and *tetA* respectively (Fig. 6).

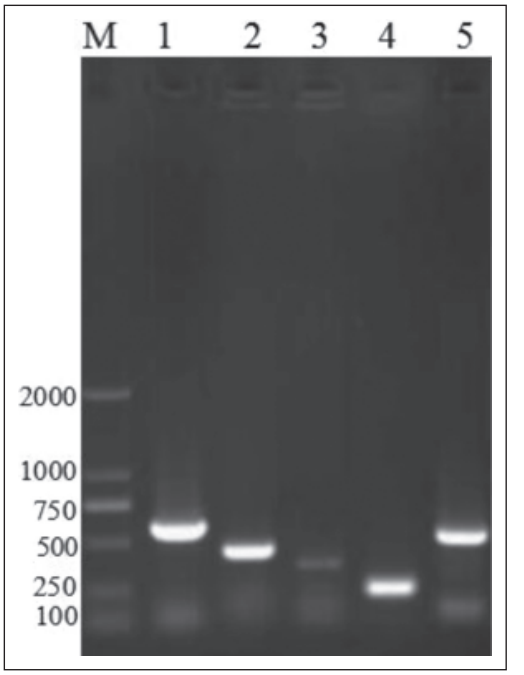

Figure 5. Amplified products of antibiotic resistance genes .
M: marker; 1: *tetM;* 2: *tetA*, 3: *aac(3)-lla*, 4: *ant(3')-la;* 5: *CTX-M*

DISCUSSION

Cattle production with 108.17 million head (National Bureau of Statistics of China, 2015) has become the 3nd largest agricultural commodity in China during past two decades (Li *et al.*, 2016). In current study, *E. coli* strains were found to be resistant to some degree to the 19 commonly utilized antibiotic drugs, especially tetracycline, cefradine, doxycycline, carbenicillin, ampicillin, ciprofloxacin and norfloxacin with resistance of more than 40.0% (Fig. 3), which is in accordance with previous studies (Yang *et al.*, 2004; Jiang *et al.*, 2011; Alonso *et al.*, 2017). Tetracycline (53.7%) resistance is predominantly observed in *E. coli* isolates, which is in line with a study in swine (Boerlin *et al.*, 2005). No resistance of *E. coli* strains against neomycin and polymyxin B was

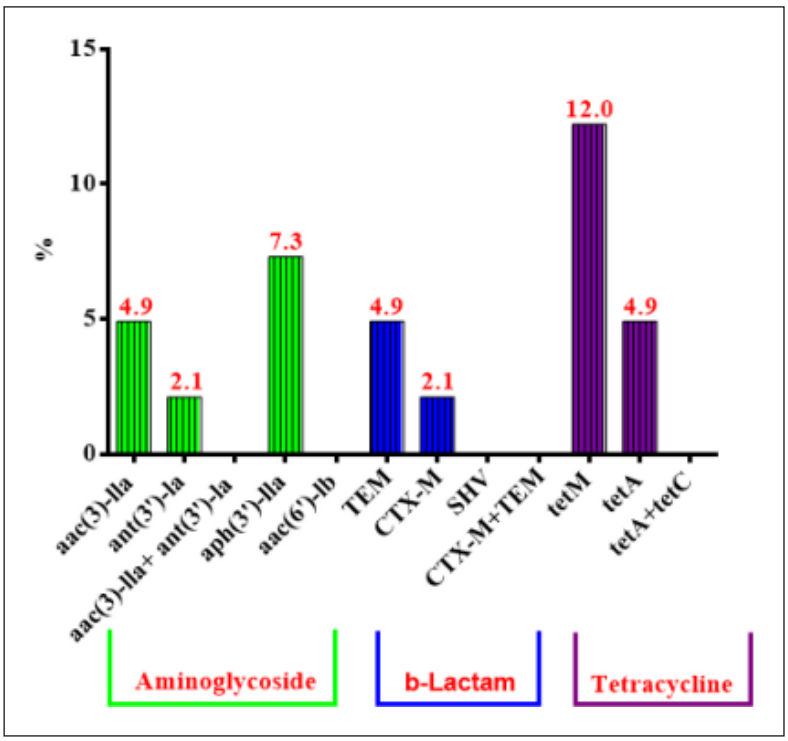

Figure 6. Antibiotic resistance genes detected in *E. coli* strains isolated from yak calves.

observed which may be due to limited collection of isolates from high remote plateau area. Multiple drug resistances were also detected in *E. coli* strains isolated from yak-calves (Fig. 4), which are concomitant with previously conducted studies about the *E. coli* resistant strains isolated from livestock (Yang *et al.*, 2004; Jiang *et al.*, 2011; Guo *et al.*, 2015; Alonso *et al.*, 2017). The present results also reveal that drug resistance; even the multiple ones, are widely distributed in *E. coli* strains isolated from diarrheal-yak calves in Hongyuan of Sichuan, China.

The extended-spectrum beta-lactamases (ESBLs) (CTX-M, SHV and TEM enzymes) are the first group of broad-spectrum b-lactamases causing resistance to b-lactam antibiotics (Pardon *et al.*, 2017). Resistant genes against aminoglycoside (aph(3')-IIa, aac(3)-IIa, aac(6')-Ib, ant(3")-Ia) and tetracycline (tet(A), tet(B), tet(C), tet(M)) were observed as plasmid-mediated resistance genes in the *E. coli* (Guo *et al.*, 2015; Navajas-Benito *et al.*, 2017). Resistant genes of aph(3')-IIa, aac(3)-IIa; ant(3")-Ia; CTX-M; and TEM, tetA and tetM were detected and found to be resistant against aminoglycoside, b-Lactam antibiotics and tetracycline, respectively (Fig. 6). In conclusion, the present study for the first time reveals the yak calves from high cold plateau as potential reservoir of *E. coli* with widely distributed multi drug resistance.

Conflict of Interest:
The authors declare that they have no competing interests.

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