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RESEARCH ARTICLE

Antibiotic Resistance Profiles and Virulence Markers of *Escherichia coli* Strains Isolated from Diarrheal Lambs in Gansu and Qinghai, China

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A B S T R A C T

The current research was done to verify the prevalence, the antimicrobial resistant strains and virulence profiles of *Escherichia coli* in sheep of western China. In total 167 lambs diarrhea samples were collected from Gansu and Qinghai province, China, during 2014-2017. A total of 103 *E. coli* isolates were subjected to verify their antimicrobial susceptibility and virulence genes. The data showed that the percentage of *E. coli* isolates was 61.68%. The main resistances were obtained for penicillin (97.09%), lincomycin (95.15%) and erythromycin (69.90%). The most common antimicrobial resistance gene was *tetA* (83.50%), *tetB* (68.93%), *blaTEM* (63.11%), *blaSHV* (56.31%) and *aac (3)-I* (78.64%). Overall, the frequently virulence genes were *mdh*, *ipaH*, *eae* and *stx1*. The most frequent combined virulence patterns were *ipaH-eae* (38.83%), *ipaH-stx1* (37.86%), *eae-stx1* (33.98%), *ipaH-astA* (23.30%), *astA-eae* (21.36%) and *ipaH-eae-stx1* (26.21%). These results demonstrated pathogenic *E. coli* are widely distributed in sheep of western China and carrying various antimicrobial resistance genes and virulence genes.

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INTRODUCTION

Antibiotics have routinely been used to control the pathogenic bacteria causing an infection in clinical medicine. However, inappropriate use of antibiotics could create selective pressures favoring resistant microbes. The selective pressures are not only to the target pathogen but also to the bystanders (Tedijanto *et al*., 2018). The antibiotic resistant bacteria and their antibiotic resistance genes increase whenever exposed to a sufficiently high antibiotic selection pressure (González-Plaza *et al*., 2018). Antibiotics can be persistent in the environment, and the rapid and ongoing spread of antimicrobial-resistant organisms may be causing a risk to public and animal health (Muhie, 2019).

Escherichia coli are ubiquitous gastrointestinal tract bacteria, and whereas most are harmless; but some types of *E. coli* are harmful that can cause many foodborne illnesses (Glowacki *et al*., 2019). *E. coli* still remains a major public health concern. Antimicrobials could contribute to antibiotic resistance development of *E. coli* by mutations of DNA and by genes that are transferred horizontally across different strains and species (Jutkina *et al*., 2018). Ruminants are the most important of antimicrobial- resistant (AMR) *E. coli* reservoir (Yamamoto *et al*., 2019). Although western China is the leader of mutton production areas, and where livestock production is the main pillar industries in agriculture, little have we known about its AMR status. Thus, the aim of this research was to characterize antimicrobial susceptibility profiles of *E. coli* isolate from feces of diarrheal lamb and identify the prevalence of genes encoding resistance to antimicrobial agents of *E. coli*.

MATERIALS AND METHODS

Sample collection and *E. coli* isolation: A total of 167 diarrheal samples were collected between January 2014 and June 2017 from intensive or semi-intensive farms (n=21) with lamb production in many cities (n=9) of Gansu and Qinghai province, China. The fresh diarrheal fecal samples were collected using sterile swabs stored in

sterilized bottles under refrigeration. The samples were incubated in nutrient broth containing 4% calf serum for 16 h at 37°C. Then, 100 µL of enriched culture was inoculated onto Eosin Methylene Blue Agar (EMBA) and MacConkey Agar (MCA) plates and incubated for 24 h at 37°C. Colonies with a green metallic sheen on EMBA and pink on MCA plates were initially considered to be *E. coli* strains.

***E. coli* strains identification:** These isolates were cultured again in nutrient broth, and DNA was extracted using bacterial DNA extraction kit (TakaRa-Bio, Dalian, China). The DNA was used to amplify 16S rDNA (TakaRa-Bio, Dalian, China) by PCR and the amplification products were analyzed by agarose gel electrophoresis. After sequencing (Beijing Genomics Institute, Shenzhen, China), BLAST was performed on obtained data.

Antimicrobial susceptibility testing: Antibiotic susceptibility testing was performed by the disk diffusion method on Mueller-Hinton agar (Guangzhou Huankai Biotechnology co. LTD, China) according to the Clinical and Laboratory Standards Institute (CLSI) protocol (CLSI, 2015). The following 15 antimicrobials were used in this study: Kanamycin (KAN), Streptomycin (STR), Gentamicin (GEN), Penicillin (PCN), Ampicillin (AMP), Meropenem (MER), Ertapenem (ERT), Polymyxin B (POB), Colistin sulphate (COS), Ciprofloxacin (CIP), Tetracycline (TET), Oxytetracycline (OXC), Chloramphenicol (CHL), Lincomycin (LIN) and Erythromycin (ERY). The antibiotic disks were all purchased from Oxoid Ltd (Basingstoke, UK). The inhibition zone of bacterial growth inhibition was measured after incubating for 24 h. Multi-drug resistant (MDR) is defined as resistance to three or more classes of antimicrobials.

Detection of resistance genes: PCR was used to detecting the resistance genes for tetracyclines (*tetA* and *tetB*), β-lactams (*blaTEM*, *blaSHV* and *blaCTX-M*) and aminoglycosides (*aac (3)-I* and *aac (6')-II*). Specific primers were selected for each of the antimicrobial resistance genes. PCR was performed with the cycling parameters reported in Table 1.

Detection of virulence genes: The following virulence genes were detected using PCR: *astA*, *stx1*, *eae*, *hlyA*, *mdh*, *ipaH*, *lt*, *bfpA and aggR*. The primers used and the conditions for amplification of these genes were described in Table 1.

Table 1: Primers used for detection of antimicrobial resistance genes (ARGs) and virulence genes

ARG/ virulence genes	Primer sequence (5'→3')	Annealing tempera-ture (°C)	Amplicon size (bp)
tetA-F	CGAAAGGCGGGCACTCAT	60	326
TetA-R	CGGCAGGCAGAGCAAGTAGAG		
TetB-F	ACGTGATAATACAGATACCGAA	55	232
TetB-R	CACAAAGGCTTGGAATACTGA		
BlaTEM-F	TTTCGTGTCGCCCTTATTC	58	692
BlaTEM-R	CCGGCTCCAGATTTATCAGC		
BlaSHV-F	CACTCAAGGATGTATTGTG	52	885
BlaSHV-R	TTAGCGTTGCCAGTGCTCG		
blaCTX-M-F	ATGTGCAGYACCAGTAA	51	536
blaCTX-M-R	ACCGCRATATCRTTGGT		
aac (3)-I-F	TTCATCGCGCTTGCTGCYTTYGA	55	239
aac (3)-I-R	GCCACTGCGGGATCGTCRCCRTA		
aac (6')-II-F	CACAGTCGTACGTTGCKCTBGG	64	235
aac (6')-II-R	CCTGCCTTCTCGTAGCAKCGDAT		
AstA-F	TGCCATCAACACAGTATATCCTCA	60	117
AstA-R	GGTCGCGAGTGACGGC		
stx1-F	ACACTGGATGATCTCAGTGG	57	605
stx1-R	CTGAATCCCCCTCCATTATG		
Eae-F	GACCCGGCAACAAGCATAAGC	62	350
Eae-R	CCACCTGCAGCAACAAGAGG		
HlyA-F	ACGATGTGGTTTATTCTGGA	54	162
HlyA-R	CTTCACGTGACCATACATAT		
Mdh-F	GGTATGGATCGTTCCGACCT	60	304
Mdh-R	GGCAGAATGGTAACACCAGAGT		
IpaH-F	AGTCTTTCGCTGTTGCTGCT	60	300
IpaH-R	AGAAACGCATTTCCTTCACG		
Lt-F	GGCGACAGATTATACCGTGC	57	360
Lt-R	CGGTCTCTATATTCCCTGTT		
BfpA-F	AATGGTGCTTGCGCTTGCTGC	62	297
BfpA-R	GCCGCTTTATCCAACCTGGTA		
AggR-F	GTATACACAAAAGAAGGAAGC	54	254
AggR-R	ACAGAATCGTCAGCATCAGC		

Table 2: Prevalence of antimicrobial resistance in *E. coli* isolates from sampling sites of Gansu and Qinghai, China

Antimicrobials		Breakpoint (CLSI, 2015) R/I/S (mm)	Percentage of resistant isolates, % (*n*=103)
Aminoglycosides	KAN	≥18/14–17/≤13	20 (19.42)
	STR	≥15/12–14/≤11	42 (40.78)
	GEN	≥15/13–14/≤12	23 (22.33)
β-lactams	PCN	≥17/14–16/≤13	100 (97.09)
	AMP	≥17/14–16/≤13	42 (40.78)
	MER	≥23/20–22/≤19	7 (6.80)
	ERT	≥22/19–21/≤18	3 (2.91)
Polypeptide	POB	≥12/–/≤11	9 (8.74)
	COS	≥11/–/≤10	2 (1.94)
Tetracyclines	TET	≥15/12–14/≤11	44 (42.72)
	OXC	≥15/12–14/≤11	45 (43.69)
Quinolones	CIP	≥21/16–20/≤15	27 (26.21)
Phenylpropanol	CHL	≥18/13–17/≤12	19 (18.45)
Lincosamides	LIN	≥21/15–20/≤14	98 (95.15)
Macrolides	ERY	≥23/14–22/≤13	72 (69.90)

RESULTS

A total of 103 (61.68%) *E. coli* were identified from the 167 samples. These colonies of the strains were mainly green metallic sheen on EMBA (Fig. 1A) and pink on MCA (Fig. 1B) plates. Gram staining showed that the strains were stained negative. Microscopic observation under 1000× magnifications showing that these strains dull circle, scattered or paired (Fig. 1C). The results of 16S rDNA PCR products are shown in Fig. 1D. The PCRs were clear of contamination from the no amplification observed in negative control. A DNA fragment was amplified from this strain with the expected length of ~600 bp (Fig. 1D). Sequence analysis showed the bacterium was *E. coli*. Its 16S rDNA shares 99% homology with that of known *E. coli* listed in the GenBank database. The antimicrobial susceptibility percentage of the *E. coli* isolates was reported in Table 2. The highest resistance rate was observed against penicillin (97.09%) and lincomycin (95.15%), followed by erythromycin (69.90%), oxytetracycline (43.69%), tetracycline (42.72%), streptomycin (40.78%) and ampicillin

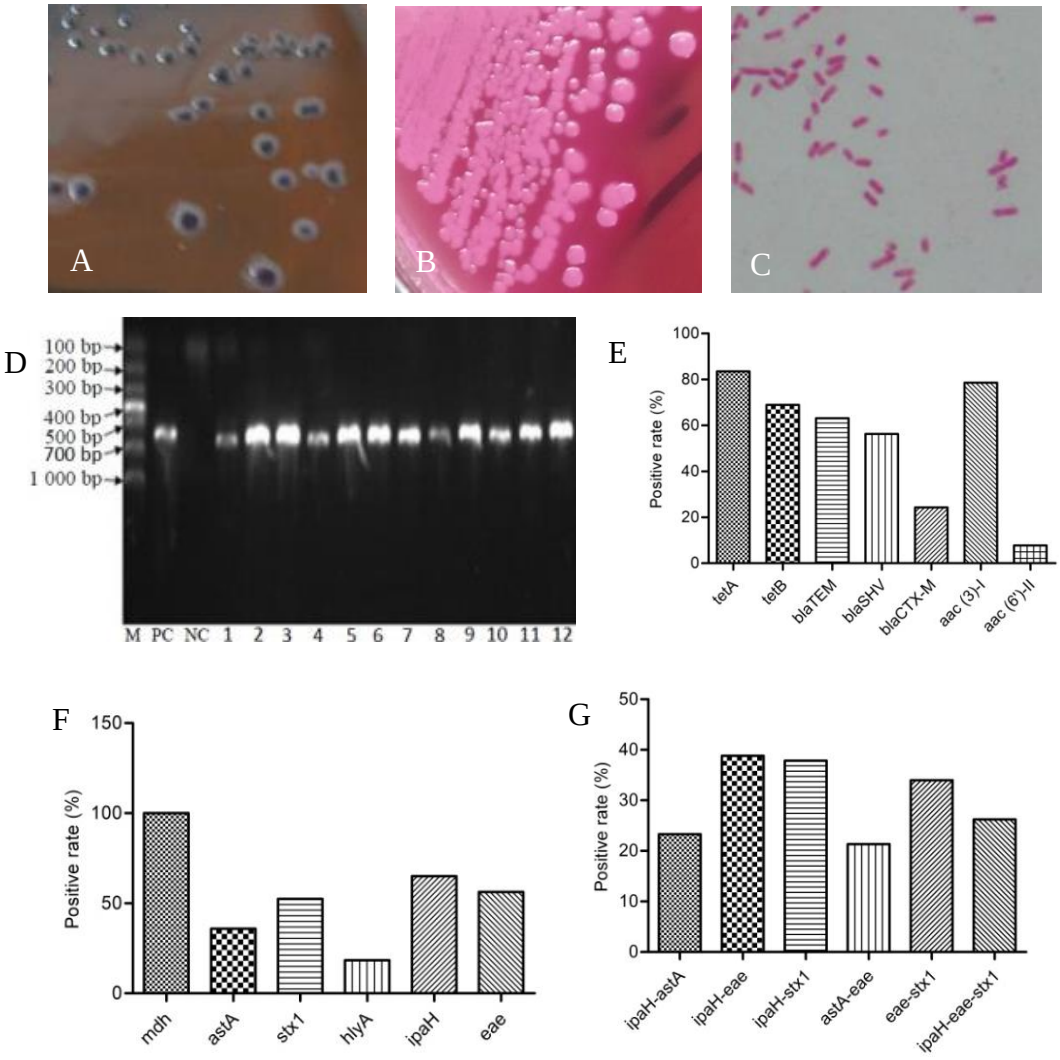

Fig. 1: The characterizations of antimicrobial resistance genes and virulence genes in *E. coli* isolates. A) Colony morphology on EMBA; B) Colony morphology on MCA; C) Gram staining result; D) 16S rDNA agarose gel electrophoresis of PCR amplified product of *E.coli*, M: 1000 bp DNA Ladder; PC: Positive control; NC: Negative control; 1~12: *E. coli*; E) Testing result of antimicrobial resistance gene; F) The high frequency of virulence genes is *mdh*, *ipaH*, *eae* and *stx1*; G) The most *E. coli* isolates were carrying two virulence genes.

(40.78%). The majority of *E. coli* isolates (*n*=88, 85.44%) were classified as MDR with predominant patterns for penicillin, lincomycin, erythromycin, streptomycin, tetracycline and ampicillin (Table 2). Among the studied antimicrobial resistance genes, *tetA*, *tetB*, *blaTEM*, *blaSHV*, *blaCTX-M*, *aac(3)-I* and *aac(6')-II* were found in 86/103 (83.50%), 71/103 (68.93%), 65/103 (63.11%), 58/103 (56.31%), 25/103 (24.27%), 81/103 (78.64%) and 8/103 (7.77%) of *E. coli* isolates, respectively (Fig. 1E). Overall, the frequency of 4 virulence genes (*mdh*, *ipaH*, *eae* and *stx1*) was >50% among all *E. coli* isolates examined. Among virulence genes, the most prevalent gene was *mdh* (100.00%), followed by *ipaH* (65.05%), *eae* (56.31%), *stx1* (52.43%), *astA* (35.92%) and *hlyA* (18.45%) (Fig. 1F). All of the clinical *E. coli* isolates were not carrying *aggR*, *lt* and *bfpA*. As for the distribution of the virulence genes, the most isolates were carrying two virulence genes (Fig. 1G). The most frequent pattern is with the presence of *ipaH-eae* (38.83%), *ipaH-stx1* (37.86%), *eae-stx1* (33.98%), *ipaH-astA* (23.30%) and *astA-eae* (21.36%) virulence genes. And among the studied genes, *ipaH-eae-stx1* were found in 27/103 (26.21%) of *E. coli* isolates.

DISCUSSION

E. coli is one of the most important zoonotic pathogens in diarrheal lamb. In the current study, a high *E. coli* isolates rate (61.68%) was observed, and the results demonstrated that *E. coli* is the main pathogenic strains for lamb diarrhea in western China.

Resistance may occur due to horizontal gene transfer from donor phages, bacteria or free DNA (Jutkina *et al*., 2018). Antibiotics can be persistent in the environment through animal manure and human wastes and may be causing a risk to human health by promoting antibiotic resistance genes and antibiotic resistant bacteria (Sharma *et al*., 2016). In the current study, a high resistance rate to penicillin, lincomycin, erythromycin, streptomycin, tetracycline and ampicillin was observed for *E. coli* isolated. And all of tested antibiotic resistance *E. coli* isolates were exhibited main mutation of *tetA*, *tetB*, *blaTEM*, *blaSHV* and *aac(3)-I*, which was associated with high levels of resistance to tetracyclines, β-lactams and aminoglycosides.

E. coli can cause significant diarrheal and extra intestinal diseases through gene gain and loss afford

pathogenic traits and ultimately lead to the pathotypes (Croxen *et al*., 2013). Acquisition of genes is generally from mobile elements such as transposons, prophages, and plasmids (Croxen *et al*., 2013). A better knowledge of the virulence markers of *E. coli* strains causing lamb diarrhea, nine virulence genes were investigated in the present study. The gene of *astA* is considered to be part of gene segment encoding enterotoxin. The report about *astA* is rarely in China. But the ratio of 35.92% was detected for *astA* in pathogenic *E. coli* strains isolated from diarrheal lambs in our study. The *stx1* and *stx2* genes are coding Shiga toxin. Most of Shiga toxin-producing *E. coli* isolate from sheep possessed the *stx1* gene alone (52.8%) in southern Brazil (Martins *et al*., 2015). In our study, 52.43% *E. coli* isolates from sheep samples were harboring *stx1* in Qinghai and Gansu, which is in accordance with previous study. The invasiveness of *E. coli* was encoded by *ipaH* gene. In this study, the detection rate of *ipaH* was as high as 65.05%, so it was inferred that the pathogenic *E. coli* isolated from Gansu and Qinghai were mainly virulent strains. The *eae* gene encodes an adhesive protein, and the detection rate was 56.31%. But none of *aggR*, *bfpA* and *lt* gene was detected in the *E. coli* isolates. The number of virulence genes carried by pathogenic bacteria can predict the type of toxin, and thus infer the pathogenic mechanism (Delbeke *et al*., 2015). The most *E. coli* isolates were carrying two virulence genes. The most frequent pattern is with the presence of *ipaH-eae* (38.83%), *ipaH-stx1* (37.86%), *eae-stx1* (33.98%) and *ipaH-eae-stx1* (26.21%) virulence genes. The results indicated that the pathogenic *E. coli* mostly contains invasive antigen, tight adhesion and Shiga toxin isolated from diarrheal lambs in Gansu and Qinghai.

Conclusions: The pathogenic *E. coli* are widely distributed in western China, and resistance to penicillin, lincomycin and erythromycin. The major antibiotics resistance genes of *E. coli* isolates are *tetA*, *tetB*, *blaTEM*, *blaSHV* and *aac (3)-I*, and frequently carrying virulence gene of *mdh*, *ipaH*, *eae* and *stx1*.

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Author contributions: XT, HW and YL conceived and designed the experiments. XT, SW, DC, FW, HSW and WY performed the experiments. XT and HW analyzed the data. HW wrote the paper. All authors have read and approved the manuscript.

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