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Prevalence and Potential Risk Factors for *Escherichia coli* Isolated from Tibetan Piglets with White Score Diarrhea

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HLD, HZ and KL conceived and designed the experiments. MUR, MK and FN contributed reagents, materials and analysis tools. HZ, YW, ZC, JL and QXW wrote the manuscript.

Key words
***Escherichia coli (E. coli)*, Pathogenic, Virulence associated genes, O-antigen serotype, Tibetan piglets.**

ABSTRACT

This study was undertaken to determine the prevalence and pathogenic potential of *Escherichia coli (E. coli)* isolated from piglets having white score diarrhea as a result of outbreak occurred in Qinghai Tibetan Plateau in 2015. A total of 81 *E.coli* were isolated from 83 fecal samples. The organisms were inoculated on MacConkey agar and EMB agar and identified via biochemical tests. Polymerase chain reaction was used to detect representative virulence factors or genes, including *E.coli* adherence factor (*K88, CS31A, afaE-8*), toxins (*estA, estB*, *Stxl, Stx2, EAST1*), pathogenicity island (*eaeA, irp2, ETT2*) and outer membrane protein (*ompA*). Moreover O-antigen serotype was tested by slide agglutination test and a mouse model was built to assess the lethality of the *E.coli* isolates through subcutaneous-infection. Out of 81 *E.coli* isolates, the most prevalent gene detected was *ompA* (90.12%), followed by *ETT2* (69.14%), *irp2* (54.32%), *EAST1* (46.91%), *CS31A* (41.98%), *estB* (19.75%), *eaeA* (14.81%), *estA* (12.35%) and *K88* (1.23%), while others were negative. The result showed that the main serotypes of *E. coli* were O8, O64, O138, O157, O139 and O141, accounting for 60 (74.04%) of all strains, while mouse subcutaneous-infection model revealed that 45(55.56%) of the isolates were "killers", 20 (24.69%) were pathopoiesia but not lead to die and 16 (19.75%) of the isolates were non virulent. This study reported the occurrence of pathogenic *E. coli* isolated from Tibetan piglets with white score diarrhea which highlights the threat of pathogenic *E.coli* in free ranging Tibetan piglets, as the local herdsmen can directly suffer a great economic loss.

INTRODUCTION

Escherichia coli (*E. coli*) is a commensal bacterium and opportunistic pathogen that is commonly found in the intestinal tracts of animals and humans (Li *et al*., 2014). However, some *E. coli* have acquired virulence genes rendering them pathogenic and can cause a variety of diseases in animals (Kaper *et al*., 2004). The major clinical manifestation of *E. coli* is severe diarrhea, dehydration, or sepsis (Gibbs *et al*., 2004), and also remained prevalent for a long-term in Tibetan pigs, with the main clinical symptoms as stunted growth or death, especially for piglets the harm was bigger.

According to biological characteristics, mechanism of action and pathogenicity, *E.coli* can be divided into seven groups, enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E.coli* (STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC) (Gyles, 1992; Kaper *et al*., 2004). In addition, it has been proposed that *EAST1* gene-positive *E. coli* might be associated with diarrhea in human (Zhou *et al*., 2002) and enterotoxigenic *E. coli* (*ETEC*) is an important cause of diarrhoea during the pre-weaning and weaning period (Ojeniyi *et al*., 1994; Nagy and Fekete, 1999). *E.coli* have many virulence factors, including adhesin, enterotoxin, shiga toxigenic and pathogenicity island which is widely distributed among diarrheic pigs (Zhang *et al*., 2007; Contrepois *et al*., 1989). Contrepois

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adhesin is the surface of some macromolecular structure components in mostly bacteria, mainly fimbriae adhesin and afimbrial adhesion, and basis of biological engraftment which is the precondition of bacteria infection usually. The major virulence genes of adhesin are *afa/drab, iha,* and *sfa/focCD etc*. (Er *et al*., 2015). Through change the absorption and secretion of intestinal cells, enterotoxin also damage the dynamic balance of intestine, and helps the fluids and electrolytes flow into enteric cavity that cause disease with watery diarrhea (Zhang *et al*., 2007), while the associated virulence factors contains *estA, estB,* and *EAST1 etc*. Shiga toxin is a kind of highly pathogenic toxin that mainly consists of *stx1*, *stx2*. Moreover, virulence Island are the virulence genes when bacterium was evolved for adaptation to the environment change, and it is closely related to the pathogenicity of *E. coli* (Cray and Moon, 1995; Agin and Wolf, 1997), the major genes contains *eaeA*, *irp2* and *ETT2*. However, outer membrane protein can help the bacteria escape the body's immune defenses, and at the same time, it can improve the *E. coli* adsorption host cells, like *ompA*.

The antigen of *E. coli* has a complex structure, and has the regional difference and diversity; somatic antigen (O) is the major antigen and until now 173 kinds of antigens are identified in *E. coli*, most of them are not pathogenic serotypes normally, and only a handful of serotypes are pathogenic for livestock and poultry (Ciosek, 1970). The serotypes of *E. coli* are more and have a large variability which makes it much difficult for the effective prevention and control of *E. coli* diseases. The weak immunity of body or if bacteria invade the parenteral tissues can cause severe diarrhea, sepsis, even cause death. It can cause the yellow scour of newborn piglets, white score diarrhea and edema diseases, it has a higher morbidity and mortality, which is a kind of important zoonoses.

Tibetan pig is relatively an ancient original indigenous breed, a rare plateau type pig in the world and is the only high altitude pasture pig breed in China (Zhang *et al*., 2017a, b; Li *et al*., 2017). With high proteins and rich amino acids, the Tibetan pig meat is an important source of income for Tibetans (Zhang *et al*., 2017a). Clinically we often find the Tibetan piglets with white score diarrhea, some piglets even died which made the harm more serious. The previous study showed that virulence genes were more frequent in isolates from cases of diarrhea than in isolates from healthy animals (Hariharan *et al*., 2004). However, there is lack of information on the prevalence of virulence factors of porcine *E. coli*. Therefore, the present study was undertaken for the first time to study the virulence potential of *E. coli* isolated from Tibetan piglets with white score diarrhea, and will have an emphasis for the public concern.

MATERIALS AND METHODS

Sample collection, isolation and identification

The present study was carried out in Nyingchi Prefecture, in southeastern Tibet that has an average height of 3100 meters, the largest continuous high elevation ecosystem. A total of 83 fresh fecal samples with white score diarrhea were collected from local farmer's piglets from July to October, 2015. After collection, all fecal samples were stored at 4°C. These samples were transported on ice to Huazhong Agricultural University for further experiment. All samples were enriched in nutrient broth, cross-inoculated in MacConkey medium, and single pink colonies were picked and purified on MacConkey medium (Hangzhou Microbial reagent Co. Ltd., Wuhan China), Pink colour colonies were taken and inoculated on Eosin methelene blue agar (EMB) (Hangzhou Microbial reagent Co. Ltd., Wuhan China) for further validation, blue-black with a metallic green sheen colonies were regarded as an *E. coli* and identified through several biochemical tests (Urease production, Catalase test, Motility, Voges proskauer, Indole production, Carbohydrate fermentation tests, Methyl red and Citrate utilization) as given in Table I. For species identification 16S rDNA sequencing (Using universal primers) was performed as suggested by Edwardsand Ewing (1972) and Wang *et al*. (2003).

Table I.- Biochemical tests used for *E. coli* identification.

Tests	Results	Tests	Results
Urease production	-	Indole production	+
Catalase test	-	Carbohydrate fermentation tests	+
Motility	+	Methyl red	+
Voges proskauer	-	Citrate utilization	-

Screening for virulence factors and genes

The *E. coli* chromosomal DNA was extracted using boiling method (Zhang *et al*., 2015). Based on previous reference (Osawa *et al*., 2006), primers shown in Table II were designed and synthesized by Wuhan Qingke Biotechnology Co. Ltd., Wuhan, China. The PCR was performed in applied thermal cycler (Applied Biosystem) using PCR kits according to the manufactures instructions (Zhang *et al*., 2017c).

The PCR reaction was performed in a 25µL mixture containing 13µL of 2× TaqPCR master mix, 1µL of each primer, 8µL of ddH_2O and 2µL of sample. The reaction condition were applied with suitable modification (shown in Table III) as previously described by Edwards and Ewing (1972).

Table II.- Characteristics of primer pairs specific for virulence factor gene in this study.

Primer	Sequence (5'-3')	Size(bp)
K88	F: GATGAAAAAGACTCTGATTGCA	841
	R: GATTGCTACGTTC AGCGGAGCG	
CS31A	F: GGGCGCTCTCTCCTTCAAC	402
	R: CGCCCTAATTGCTGGCGAC	
afaE-8	F: CTAACTTGCCATGCTGTGACAGTA	302
	R: TTATCCCCTGCGTAGTTGTGAATC	
estA	F: TCCGTGAAACAACATGACGG	244
	R: ATAACATCCAGCACAGGCAG	
estB	F: GCCTATGCATCTACACAATC	278
	R: TGAGAAATGGACAATGTCCG	
Stxl	F: ATTCGCTGAATGTCATTCGCT	664
	R: ACGCTTCCC AG AATTGCATTA	
Stx2	F: GAATGAAGAAGATGTTTATAGCGG	281
	R: GGTTATGCCTCAGTCATTATTAA	
EAST1	F: ATGCCATCAACACAGTATATC	117
	R: TCAGGTCGCGAGTGACGG	
eaeA	F: AAGCGACTGAGGTCACT	384
	R: ACGCTGCTCACTAGATGT	
irp2	F: AAGGATTCGCTGTIACCGGAC	287
	R: TCGTCGGGCAGCGTTTCTTCT	
ETT2	F: CTTCTTCCTAACGAAACATCATTAC	913
	R: TGACATATCAACTTTCTCTTACGC	
ompA	F: AGCTATCGCGATTGCAGTG	919
	R: GGTGTTGCCAGTAACCGG	

Escherichia coli *serotyping antisera against 'O' antigens*

Slide agglutination test: The antigen (*E. coli*, 0.025 ml) and polyvalent serum (0.025ml) were mixed together on glass plate, and observed for 2 min to determine the result. At the same time, the antigen (*E. coli*, 0.025 ml) and 0.5% phenol saline were also mix together as a negative control, with no agglutination after 2 min. Positive: if more than 50% of antigen agglutinates; negative: no agglutination.

Table III.- The positive of the virulence gene.

Gene	n	No. positive	Prevalence % (95% CI)
K88	81	1	1.23 (0-6.7)
CS31A	81	34	41.98 (31.1-53.5)
afaE-8	81	0	0
estA	81	10	12.35 (6.1-21.5)
estB	81	16	19.75 (11.7-30.1)
Stx1	81	0	0
Stx2	81	0	0
EAST1	81	38	46.91 (35.7-58.3)
eaeA	81	12	14.81 (7.9-24.4)
irp2	81	44	54.32 (42.9-65.4)
ETT2	81	56	69.14 (57.9-78.9)
ompA	81	73	90.12 (81.5-95.6)

Mouse lethality assay

To assess the lethality of the *E. coli* isolates, a mouse subcutaneous-infection model was used. This model included 81 group, every group includes three treatments (standard strain C83907: killed all mice by 7 days postchallenge; negative control: no mice was killed by 7 days postchallenge; *E.coli*: under test), all mice were injected standardized bacterial inoculum (109 cfu/mL log-phase bacteria in 0.2 mL Ringer solution) subcutaneously, in the abdomen. In this model, lethality is a clear-cut parameter; when the sample and the positive control was killer, meanwhile the negative control was not killer, it is classified as killer (high virulence); the positive control was killer, the sample was pathopoiesia but not lead to die and the negative control was not killer, it is classified as moderate virulence; the positive control was killer but the sample and the negative control was not killer, it was established as non-killers (avirulence) (Picard *et al*., 1999), otherwise invalidation.

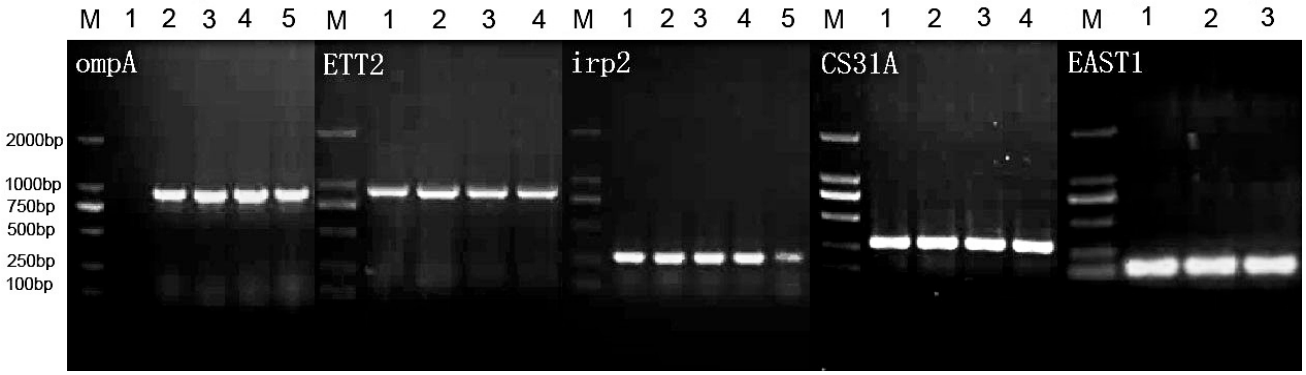

Fig. 1. PCR amplification results of the main genes. (M, marker; *ompA*, 919bp; *ETT2*, 913bp; *irp2*, 287; *CS31A*, 402bp; *EAST1*, 117bp).

RESULTS

Isolation, culturing and identification of E.coli

A total of 81 isolates were identified from 83 fresh fecal samples with white score diarrhea and the isolation rate of *E. coli* was 97.59%.

Virulence associated factor

In this study, 1(1.23%) and 34(41.98%) isolates were positive for *E. coli* adherence factor *K88* and *CS31A* gene, while *afaE-8* was negative; meanwhile 10(12.35%), 16(19.75%) and 38(46.91%) isolates were positive for toxins *estA, estB* and *EAST1* genes, respectively, while *Stxl* and *Stx2* were test negative; 12(14.81%), 44(54.32%) and 56(69.14%) isolates were positive for pathogenicity island *eaeA, irp2* and *ETT2* genes, respectively; while 73 (90.12%) isolates were positive for outer membrane protein *ompA* genes (Fig. 1).

The detection also showed that the distribution of toxin and adhesion factor is variable in 81 strains of *E. coli*, some strains having at least two adhesion factor or two virulence genes. But there were some *E. coli* that did not detect any adhesion factor or any virulence genes.

Serotyping antisera against 'O' antigens

The test showed that the main sero-types of O8, O64, O138, O157, O139 and O141 were 15(18.52%), 12(14.81%), 10(12.35%), 9(11.11%), 7(8.64%) and 7(8.64%), respectively, accounting for 60(74.07%) of all strains. The positive of O115, O161, O125, O9, O145 and O45 were very low which account for 4(4.94%), 3(3.70%), 3(3.70%), 1(1.23%), 1(1.23%) and 1(1.23%), respectively. The 8(9.88%) strains sero-types were not sure (Fig. 2).

Mouse lethality assay

Mouse subcutaneous–infection model revealed that 45(55.56%) of the isolates were "killers", 20(24.69%) were pathopoiesia but not lead to die and 16(19.75%) of the mouse do not have any symptom, and all the 45 killer isolates were positive for virulence genes.

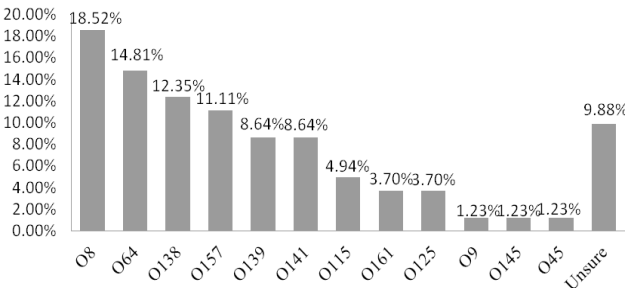

Fig. 2. The distribution of O-antigen serotype.

DISCUSSION

Infectious diseases have been serious threat for animal health and productivity in developing countries (Elhaig *et al*., 2016; Qayyum *et al*., 2016; Wen *et al*., 2016; Yilmaz *et al*., 2016). *E. coli* is the most common commensal bacterial etiologic agent of colibacillosis. The virulence genes pathogenic *E. coli* mainly include *adhesin, enterotoxin, shiga toxin* and virulence island; at first, the fimbriae of adhesin will adhere to intestinal epithelial cells and then secrete one or more than one kind of enterotoxin to make the electrolyte balance changed or injure the intra-intestinal blood vessels to cause disease or recessive infection (Imberechts *et al*., 1992).

This study showed that the virulence genes were *ETT2, irp2* and *eaeA* in *E. coli* which were isolated from Tibetan piglets with white score diarrhea. Among these virulence genes, Osawa *et al*. (2006) had proved that *ETT2* is one of the important virulence genes which cause diarrhea, it not only has close relation with *ETEC*/*STEC*, but also widely exist in other pathogenic *E. coli*; in addition, *eaeA* can adhere to the intestinal epithelial cell, causing diarrhea and only detection of *irp2* gene level can be used as an indicator of the possible pathogenic capability of *E. coli*. *Irp2* is the iron regulatory gene which only express in pathogenic *E. coli*, the level of *irp2* gene can be used as an indicator of the pathogenic capability of *E. coli*. *CS31A* is the fimbriae protein in *E. coli* which can also adhere to the intestinal epithelial cells, playing a big role in the pathogenic process. *ompA* makes permeability for outer membrane and maintain the stability of outer membrane structure. The highest prevalence of *ompA* is in complete agreement with the findings of Ashraf *et al*. (2014) in humans and chickens. In aggrement to our results another researcher Hariharan *et al*. (2004), who found the same prevalence of *E. coli* virulence associated genes in piglets with diarrhea and suggested the presence of association between virulence genes and cases of diarrhea.

Some serotype are also related to the pathogenicity in *E. coli*, such as O101, O147, O139, O157, O141 and O149, *etc*. (Garabal *et al*., 1996; Ojeniyi *et al*., 1994). Now immunization is the main way to prevent the *E. coli* disease, due to more serotype, complexity of antigen and the high variability characteristics in *E. coli*, moreover, serotype exist certain differences in different areas, it brings much difficulties to the prevention and control of the *E. coli* diseases.

The prevention and control of *E. coli* diarrhea except testing the distribution of the main virulence factors, survey superiority serotype in order to choose appropriate vaccine is also indispensable in the region. The study showed that the advantage of serotype in Tibetan piglets

with white score diarrhea were O8, O64, O138, O157, O139 and O141 which was same with most parts of China (Sun *et al*., 2004). However, some research showed the different results (Li *et al*., 2000), the probable reason may be that these serotypes are relatively popular in Tibet or the number of samples tested were small which can't represent the entire area on serum type characteristics in Tibetan pigs, and need further research.

Tibetan pig is widely known for its tolerance to disease and strong adaptability to the harsh Tibetan environment of low oxygen levels and changeable temperatures (Li *et al*., 2005). But this research has showed that there are still many piglets with the *E. coli* disease, the main reasons might be that management does not reach the designated position, weak consciousness of prevention and controling disease in local farmers and herdsmen or the high altitude and large temperature difference between day and night, which can easily drop the immunity level of piglets, causing the occurrence of *E. coli* disease. In addition, the soil and water contaminated by the feces in the environment is the important reason that lead to the diarrhea; moreover, nutritional factors, sanitary conditions and environmental factors can also lead to this condition. In this region free grazing and feeding systems are adopted therefore, more liquidity among pigs thus increases the spreading opportunity for *E. coli* diseases (Johanna *et al*., 2014).

It is suggested that, so herein we suggest some measures should be taken prevent and control the *E. coli* infection. Initially, it is important to improve the management of swine breeding to decrease spread of disease from direct contact between healthy and diarrhea pigs. Furthermore, regular disinfection is necessary to use for piggery and isolation of infected pigs correspondingly during outbreak of disease. Lastly, authorities should set a strict policy for the prevention and control of disease to spread from one place to another.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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