Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Antimicrobial Resistance of *Escherichia coli* Isolated from Tibetan Piglets Suffering from White Score Diarrhea

Hui Zhang[1§], Mujeeb Ur Rehman[1§], Kun Li[1], Houqiang Luo[1,3], Yanfang Lan[1], Fazul Nabi[1], Muhammad Shahzad[4], Shucheng Huang[1], Xinyu Liu[1], Khalid Mehmood[1,4], Muhammad Kashif Iqbal[1] and Jiakui Li[1,2*]

[1]College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, People's Republic of China; [2]Laboratory of Detection and Monitoring of Highland Animal Disease, Tibet Agriculture and Animal Husbandry College , Linzhi 860000 Tibet, People's Republic of China; [3]Animal Science Department, Wenzhou Vocational College of Science & Technology 325006, People's Republic of China; [4]College of Veterinary & Animal Sciences, The Islamia University of Bahawalpur 63100, Pakistan
*Corresponding author: lijk210@sina.com

ARTICLE HISTORY (16-105)

Received: April 30, 2016
Revised: October 19, 2016
Accepted: October 30, 2016
Published online: November 23, 2016

Key words:
Antimicrobial resistance
Escherichia coli (E. coli)
Tibetan piglets
Tolerance
White score diarrhea

A B S T R A C T

The aim of this study was to investigate the drug resistance profile of *Escherichia coli* (*E. coli*) isolated from Tibetan piglets having white score diarrhea in Qinghai Tibetan Plateau of China. A total of 81 *E. coli* strains were isolated from 83 fecal samples. While commonly used antimicrobial drugs and polymerase chain reaction (PCR) were used to detect the drug resistant and representative drug resistance genes. The results showed that the isolated *E. coli* was highly resistant to medemycin (100%), aztreonam (87.65%), tobramycin (55.56%), and kanamycin (46.91%), and lower resistant to cefoxitin (40.74%) and ceftazidime (30.86%). Isolates tolerant to 3 or more than 3 antimicrobial drugs accounted for 87.65% and were classified as multidrug resistant (MDR). The prevalent genes detected were bla_{TEM} and *aac-Im*, belonging to *beta-Lactamases* and *aminoglycosides*, class respectively. In conclusion, the antimicrobial resistance of *E. coli* from free-ranging Tibetan piglets showed a high tolerant rate than other regions of China. The results of the present study revealed that, the use of single antibiotic for a long period of time was the main reason that increased the antibiotic resistance in Tibetan pigs. Therefore, the insensitive antimicrobial drugs should be avoided in clinical treatment of bacterial diseases.

To Cite This Article: Zhang H, Rehman MU, Li K, Luo H, Lan Y, Nabi F, Shahzad M, Huang S, Liu X, Mehmood K, Iqbal MK and Li J, 2017. Antimicrobial resistance of *Escherichia Coli* isolated from tibetan piglets suffering from white score diarrhea. Pak Vet J, 37(1): 43-46.

INTRODUCTION

Escherichia coli (*E. coli*) is a commensal bacterium and opportunistic pathogen that is commonly found in the intestinal tracts of animals and humans (Agin *et al.,* 1997). Pathogenic *E. coli* is important intestinal pathogen in both humans and animals, including mainly enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC) (Ojeniyi *et al.,* 1994; Nagy *et al.,* 1999). *E. coli* is generally used as an indicator to observe anti-microbial resistance in livestock and meat industry (Moyaert *et al.,* 2014). Pathogenic *E. coli* can cause severe diarrhea, and it is an important reason for large among of deaths of swine and huge economic losses (Tian *et al.,* 2011). White scour diarrhea of piglets is a clinically common acute and highly lethal intestinal infectious disease caused by enterotoxigenic *E. coli* (ETEC). After the accomplishment of this infection in piglet, the rate of mortality and medicinal cost increases significantly. The poor growth performance, high morbidity rate and estimated 50% of swine mortality is due to diarrhea infections (Morris *et al*., 2002; Jin *et al*., 2000).

Antimicrobial agents are widely used in livestock and poultry industry. The misuse of antimicrobial agents leads to the high level of bacterial resistance, alongside the emergence of multiple antibiotic resistant strains; an increase in the global health risk of antimicrobial agents also occurs (Levy and Marshall, 2004). *E. coli* normally lives and reproduces in the intestinal flora as an opportunistic pathogenic bacterium and widely exists in the

[§]First two authors made equal contributions to this article.

environment. The research to evaluate the selective impact on *E. coli* species to various antimicrobial agents revealed that the selective pressure exerted by these agents is impressive and cumulative (Diarrassouba *et al*., 2007).

The mechanism of microbial resistance is based upon genetic flexibility of bacteria, due to over use of antimicrobial agents that leads to multiple drug resistance and spread of antimicrobial resistance globally in humans, animals and agriculture medicine (Paulo *et al*., 2013).

Tibetan pig is relatively an ancient unique indigenous breed, a rare plateau type pig in the world and one of the only high altitude pasture pig breed in China (Lan *et al.*, 2016; Li *et al*., 2016). It is mainly found in the Yarlung Zangbo river valley and eastern region of the Tibet. Tibetan pig becomes an important source of income for Tibetans nomads due to strong adaptability to harsh Tibetan environment of low oxygen levels and variable temperatures. The Tibetan breeds are famous for high proteins and rich amino acids diet; furthermore, this breed has the ability to tolerate several diseases with its unique immunity characteristics (Xin *et al*., 2011; Zhang *et al*., 2014). A researcher from China has reported a high prevalence of anti-microbial resistance (Li *et al*., 2014) in Tibetan pigs but until now very less information is available about the molecular mechanism of drug resistance in Tibet region of China. Since mostly antibiotic therapy are used to treat the pig diseases, which leads to a number of high antimicrobial resistance levels and multiple drug resistance phenomenon in Tibetan pigs. Antimicrobial resistance is a worldwide health problem and free ranging farming system may contribute risk of formation of antimicrobial burden in Tibet autonomous region of China. Therefore, this experiment was carried out to investigate antimicrobial usage and to explore the drug resistance in *E. coli* from Tibetan piglets with white score diarrhea and to provide the information for the prevention and control of the *E. coli* infection in swine.

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 (Fig 1). A total 83 fresh fecal samples with white score diarrhea was collected from free-ranging piglets from July to October, 2015. All the sampling was done randomly with irrespective of the gender. After collection, all fecal samples were stored at 4°C. These samples were transported on ice to Huazhong Agricultural University for further experimentation. Fecal samples were cultured on nutrient broth, then organism was cross-inoculated on MacConkey agar (Hangzhou microbial, China), and single pink color colonies were picked and purified on MacConkey agar. Pink color colonies were taken and inoculated on selective media i.e. Eosin methylene blue agar (EMB) (Hangzhou microbial, China), suspected colonies were subjected to gram staining (first in gram dye solution after dyeing, with 100x optical show Micro mirror for microscopy, according to the bacterial form, and the characteristics of the staining, the gram staining was negative, both ends obtuse, no spores and medium-sized coli preliminary as *E. coli*). For further validation, blue-black color colonies with a metallic green sheen color 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) (Table 1) (Edwards and Ewing, 1972; Tabar *et al*., 2016). For species identification 16S rDNA sequencing (Using universal primers) was performed as suggested by Edward and Wang (Edwards *et al*., 1972; Wang *et al*., 2003).

Antibiotic sensitivity tests: Disk diffusion test was performed to detect antibiotic sensitivity of the isolates by using (NCCLS, 1999) following drugs: quinolone class: ciprofloxacin, ofloxacin and norfloxacin; amino sugars nucleoside: gentamycin, tobramycin, kanamycin and streptomycin; beta-Lactam class: cefoxitin, cefalotin, ceftriaxone, cefuroxime, ceftazidime, cefotaxime, cefoperazone, ampicillin and aztreonam; tetracycline; macrolide class: medemycin (Hangzhou Microbial, China).

In this experiment, standard strain of bacteria ATCC25922 strain (HZAU, Wuhan) was used as control positive while clear broth as negative control. Results of resistance (R), intermediate (I) and sensitive (S), were recorded according to criteria of clinical laboratory standards committee (CLSI, 2014).

The sensitivity of the antibiotics was also confirmed by using broth dilution method following the guidelines and standards provided by NCLLS. At the same time, ATCC25922 was used as a quality control. Each drug test was repeated 3 times to avoid muddy hole corresponds. The lowest drug concentration was used as the minimum bacteriostatic concentration of the corresponding drug.

Screening for drug resistance genes: The *E. coli* chromosomal DNA was extracted using boiling method (Zhang *et al*., 2015). Briefly, the samples were inoculated into APW-A, and incubated broth was centrifuged at 8000rpm for 10min, and the supernatant was discarded. 50μL of sterile distilled water was added to the tubes and boiled in a water bath at 100°C for 10min and immediately transferred onto ice.

Based on previous reference (Joseph, 2001; Vincent, 2005; Olson, 2005; Karami, 2008; Mao, 2011; Zheng, 2013), primers shown in Table 2 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. The PCR reaction was performed following standard protocol (Zhang *et al*., 2012). The PCR products were separated through electrophoresis in 1.5% (w/v) agarose gel stained with ethidium bromide and observed with gel imaging system purchased (Gene Genius BioImaging System, UK).

RESULTS

Isolation, culturing and identification of *E. coli*: A total 83 fresh fecal samples with white score diarrhea were collected and 81 isolates of *E. coli* were identified, with overall isolation rate of 97.59%.

Antibiotic resistance of *E. coli* isolates: In present study among 81 *E. coli* isolates from Tibetan piglets with white

Fig. 1: Collection site of the samples in Tibetan piglets, Tibet China.

Table 1: Biochemical tests used for *E. coli* identification

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

Table 2: The sequence and oligonucleotide primers for the PCR and the sizes amplified

Primer	Sequence (5'-3')	Size(bp)	References
bla_{TEM}	F: TCGCCGCATACACTATTCTCAGAATGA R: ACGCTCACCGGCTCCAGATTTAT	445bp	Mao, 2011
bla_{CTX-M}	F: ATGTGCAGYACCAGTAARGTKATGGC R: TGGGTRAARTARGTSACCAGAAYCAGCGG	593bp	Olson, 2005
bla_{OXA-1}	F: ATATCTCTACTGTTGCATCTCC R: AAACCCTTCAAACCATCC	619bp	Karami, 2008
bla_{SHV}	F: GGGTTATTCTTATTTGTCGC R: TTAGCGTTGCCAGTGCTC	567bp	Vincent, 2005
qnrA	F: ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTTAGGTCA	730bp	Zheng, 2013
qnrB	F: GATCGTGAAAGCCAGAAAGG R: ACGATGCCTGGTAGTTGTCC	469bp	Zheng, 2013
aac-Im	F: GGCTGACAGATGACCGTGTTCTTG R: GTAGATATTGGCATACTACTCTGC	303bp	Joseph, 2001

score diarrhea, all the isolates were sensitive to ceftriaxone, cefoperazone, ofloxacin and ofloxacin. For other antibiotics, including norfloxacin, cefalotin, streptomycin, cefotaxime, cefuroxime, the number of isolates were 3, 4, 5, 8 and 15, respectively. The highest resistance was found for medemycin (81 isoaltes), followed by aztreonam, tobramycin, kanamycin, cefoxitin (33 isolates), ceftazidime, ampicillin, gentamycin and tetracycline (Fig. 2). A total of 71 MDR strains were isolated from piglets. The rates of multidrug resistant (MDR) *E. coli* isolates are summarized in Fig. 3.

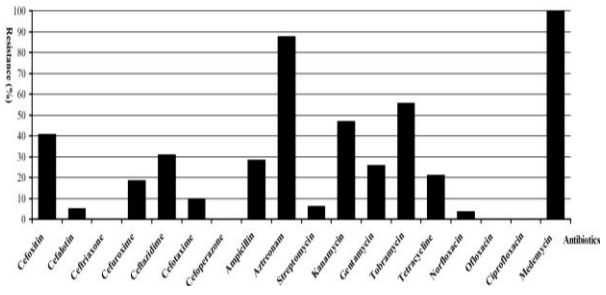

Fig. 2: Antimicrobial sensitivity testing of *E. coli* isolates from Tibetan pigs.

PCR detection of drug resistance genes: The results showed that 34.57 and 22.22% *E. coli* isolates were positive for bla_{TEM} and aac-Im genes respectively, while bla_{CTX-M}, bla_{OXA-1}, bla_{SHV}, qnrA, qnrB genes were negative for all the tested isolates (Fig. 4).

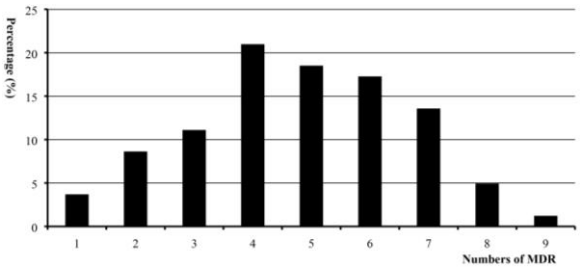

Fig. 3: The results of multi-drug resistance (MDR).

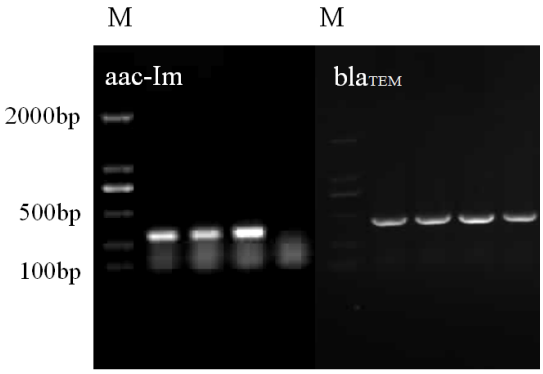

Fig. 4: The PCR amplification results of the aac-Im and bla_{TEM}.

DISCUSSION

E. coli post weaning diarrhea is very common cause of huge economic losses in pig production. It increases mortality and morbidity rate and cost of medication and decreases the growth performance in pig industry (Nagy and Fekete, 2005). One specific serotypes of *E. coli* can cause various infections in piglets, however there is no pertinent vaccine to prevent it, although the antimicrobial agent is often used for the control and treatment of various colibacillosis diseases. Recently, due to increase use of antimicrobial agents, the bacteria have developed resistance against the several antibacterial agents (Fred, 2006). Generic *E. coli* is the gram-negative bacteria, which develops resistance to antibiotics easily; at the same time, the resistances within the bacteria become complex which leads to multi drug resistant strains of the organism which may cause many difficulties in controlling the diseases (Threfall *et al*., 1993; Varga *et al*., 2008).

Considering the serious state of indiscriminate use of antibiotics, many people have paid attention to drug-resistant strains (Zhang *et al*., 2012). The resistance to *E. coli* is associated with the use of antimicrobial agents in veterinary medicine, and the drug resistance of *E. coli* bacterium is different in different region (Wang *et al*., 2013). Although in the same area there still have different resistance strains in different habits. Therefore, it is very important to investigate the drug resistance of *E. coli* in Tibetan region within the scope of pigs. In this study, we investigate the antimicrobial resistance of *E. coli* isolated from free-ranging Tibetan piglets with white score diarrhea for determining the resistance of colibacillosis in Tibetan piglets and providing a positive reference to the treatment of colibacillosis outbreak in the future.

In the present findings, the rate of resistance to antibiotics was relatively low in the Tibetan piglets *E. coli* isolates than some other regions of northeast (Liaoning

province), north (Beijing), southwest (Sichuan province), southern (Guangdong province) and central (Henan and Jiangxi provinces), regions of China (Lei *et al*., 2008). Moussa *et al* (2007) also reported low rates of resistance to ceftiofur, streptomycin, and gentamicin in chickens than those receiving feed with the corresponding antimicrobials (Moussa *et al*., 2007). Present results revealed that free ranging Tibetan pigs were exposed to antibiotics found in the specific area, which is one of the important reason that have a lower resistance to antibiotics in Tibetan piglets. For further confirmation of the results, the drug susceptibility test was confirmed by PCR for the tested β-lactams and aminoglycoside antibiotics. In our study, the rates of resistance to commonly used β-lactams and aminoglycoside were 34.57 and 22.22%, respectively, while the sulfonamides were found to be negative, alongside the drug sensitivity test have found the similar phenomena. An important aspect of the drug resistance in Tibetan area may be due to rational use of antibiotics by the farmers and then using same kind of antibiotics for long-time. In addition, China's Tibet Autonomous Region is a famous tourism hotspot with hyper mobility, which may severe as a risk for the dissemination of antibiotic resistance.

Conclusions: Our findings demonstrate the antimicrobial resistance of *E. coli* in free-ranging Tibetan piglets with white score diarrhea. Therefore, it is suggested to use drug sensitive test to determine the suitable dose. Furthermore, the husbandry of Tibetan pigs and diagnosis techniques for bacterial infection needs to be updated to decrease antimicrobial resistance in Tibetan pigs. Additionally, authorities should strictly make a policy for the control and safe use of antibiotic drugs in veterinary medicine.

Acknowledgements: This study was supported by Key Science Fund of Science and Technology Agency of Tibet Autonomous Region, Twelfth Five-Year National Science and Technology Support Project (Grant No. 2012BAD3B03) and Chinese Agricultural Research Systems (CARS-38).

Author contributions: JKL and HZ conceived and designed the experiments; HZ and MUR performed the experiments; KL, YFL, HQL, FN, MS, MKI, XYL, MK and SCH contributed reagents, materials, and analysis tools; HZ and JKL wrote the manuscript.

REFERENCES

Agin TS and Wolf MK, 1997. Identification of a family of intimins common to *Escherichia coli* causing attaching-effacing lesions in rabbits, humans, and swine. Infect Immun 65:320-6.

CLSI, 2014. Performance standards for antimicrobial susceptibility testing; Twenty-First Informational Supplement. CLSI/NCCLS M100-S24. Clinical and Laboratory Standards Institute, Wayne, PA.

Paulo MDC, Luís L and Augusto JFM, 2013. Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment. Int J Environ Res Public Health 10:278-94.

Diarrassouba F, Diarra MS, Bach S, *et al*., 2007. Antibiotic resistance and virulence genes in commensal *Escherichia coli* and salmonella isolates from commercial broiler chicken farms. J Food Protect 70:1316-27.

Edwards PR and Ewing WH, 1972. Identification of Enterobacteriaceae. 3rd Ed. Burgess Publishing Company, Minneapolis, USA, pp:1-107.

Feng W, Wang XQ, Zhou W, *et al*., 2011. Isolation and characterization of lipase-producing bacteria in the intestine of the silkworm, Bombyx mori, reared on different forage. J Insect Sci 11:135.

Fred C and Tenover, 2006. Mechanisms of antimicrobial resistance in bacterial. Am J Med 119:3-10.

Jin LZ and Zhao X, 2000. Intestinal receptors for adhesive fimbriae of enterotoxigenic *Escherichia coli* (ETEC) K88 in swine-a review. Appl Microbiol Biotechnol 54:311-8.

Lan YF, Li K, Zhang H, *et al*., 2016. Prevalence of high-level aminoglycoside resistant *Enterococci* isolated from Tibetan pigs. Pak Vet J 36:503-5.

Lei D, Li ML, Wu CM, *et al*., 2008. Characterization of antimicrobial resistance among *Escherichia coli* isolates from chickens in China between 2001 and 2006. FEMS Microbiol Lett 286:178-83.

Levy SB and Marshall B, 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat Med 10:122-9.

Li K, Luo HQ, Zhang H, *et al*., 2016. First report of *Metastrongylus pudendotectus* by the genetic characterization of mitochondria genome of cox1 in pigs from Tibet, China. Vet Parasitol 223:91-5.

Li P, Wu DF, Liu KY, *et al*., 2014. Investigation of antimicrobial resistance in *Escherichia coli* and *Enterococci* isolated from Tibetan pigs. PLoS ONE 9:e95623.

Morris RS, Davies PR and Lawton DE, 2002. Evolution of diseases in the world's pig industry. 17th International Pig Veterinary Society Congress Proceedings; Ames, Iowa, pp:1-10.

Moussa SD, Fred GS, Fatoumata D, *et al*., 2007. Impact of feed supplementation with antimicrobial agents on growth performance of broiler chickens, clostridium perfringens and *Enterococcus* counts, and antibiotic resistance phenotypes and distribution of antimicrobial resistance determinants in *Escherichia coli* isolates. Appl Environ Microbiol 20:6566-76.

Moyaert H, Jong AD, Simjee S and Thomas V, 2014. Antimicrobial resistance monitoring projects for zoonotic and indicator bacteria of animal origin: common aspects and differences between EASSA and EFSA. Vet Microbiol 171:279-83.

Nagy B and Fekete PZ, 1999. Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. Vet Res 30:259-84.

Nagy B, Fekete PZ, 2005. Enterotoxigenic *Escherichia coli* in veterinary medicine. Int J Med Microbiol 295:443-54.

National Committee for Clinical Laboratory Standards, 1999. Performance standards for antimicrobial susceptibility testing. Ninth informational supplement M100-S9. Wayne: NCCLS, pp:32-75.

Ojeniyi B, Ahrens P and Meyling A, 1994. Detection of fimbrial and toxin genes in *Escherichia coli* and their prevalence in piglets with diarrhoea. The application of colony hybridization assay, polymerase chain reaction and phenotypic assays. J Vet Med 41:49-59.

Xin SP, Shi D and Zhang J, 2011. Research of protection, development and utilization of genetic resources of Tibetan pig in Tibet Autonomous Region. Chin Anim Husbandry Bull 4:49-51. (in Chinese)

Threfall EJ, Rowe B and Ward LR, 1993. A comparison of multiple drug resistance in *Salmonellas* from humans and food animal in England and Wales, 1981 and 1990. Epidemiol Infect 111:189-97.

Tian GB, Wang HN, Zhang AY and Zhang Y, 2011. Detection of resistance to β-lactams and characterization of extended-spectrum lactamases in *Escherichia coli* isolates from swine. Chin. J Prev Vet Med 10:776-80.

Varga C, Rajie A and MeFall ME, 2008. Comparison of antimierobial resistance in generic *Escherichia coli* and *Salmonella* spp. Cultured from identical fecal samples in finishing swine. Can J Vet Res 72:181-7.

Wang CG, Lv JC and Zhang T, 2013. Detection of resistance phenotype and genotype of avian *Escherichia coli* in Hebei Province. Poultry Sci 92:2326-32.

Wang X, Heazlewood SP, Krause DO and Florin THJ, 2003. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16s rDNA sequence analysis. J Appl Microbiol 95:508-20.

Zhang NZ, Zhou DH, Huang SY, *et al*., 2014. Seroprevalence and risk factors associated with *Haemophilus parasuis* infection in Tibetan pigs in Tibet. Acta Tropica 132:94-7.

Zhang T, Wang CG, Lv JC, *et al*., 2012. Survey on tetracycline resistance and antibiotic-resistant genotype of avian *Escherichia coli* in North China. Poultry Sci 91:2774-7.

Zhang YX, Han XG, Zuo SK, *et al*., 2015. Distribution of lipopolysaccharide core type in avian pathogenic *Escherichia coli* and its correlation with virulence gene. Microbiology (China) 42:1619-25.