Genotypic and Phenotypic Characterization of Antimicrobial-Resistant *Escherichia coli* from Farm-Raised Diarrheic Sika Deer in Northeastern China

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Abstract

In China, overuse and/or abuse of antimicrobials are common in stockbreeding, which possess high risks of antimicrobial-resistant contaminations. The serogroups, major virulence genes, and antimicrobial resistant patterns of the antimicrobial-resistant *Escherichia coli* (*E. coli*) were investigated in the feces of diarrheic farm-raised sika deer from 50 farms in three Northeastern provinces of China. A total of 220 *E. coli* isolates were obtained and characterized. Twenty-eight O serogroups were identified from the obtained *E. coli* isolates with O2, O26, O128, O142 and O154 being dominant. Nearly all the isolates were resistant to at least four of the tested antimicrobials. More than 90% of the *E. coli* isolates carried at least one of the tested virulence genes. About 85% of the *E. coli* isolates carried one or more antimicrobial-resistant genes responsible for resistant phenotypes of sulfonamides, streptomycin/spectinomycin or tetracycline. The antimicrobial resistant level and pathogenic group occurrences of the obtained *E. coli* isolates were higher than that of livestock and wild animals reported in some developed countries. Thus, the fecal-carrying antimicrobial-resistant *E. coli* from the farm-raised sika deer is potentially a significant contamination source for freshwater systems and food chain, and may pose great health risks for human and animals in Northeastern China.

Introduction

Antimicrobial resistance (AMR) in enteropathogens has become a major public health problem due to its potential infections on human and animals [1]. The domestic animals are usually considered as major reservoirs for antimicrobial-resistant bacteria. Recently, increasing interest has been given to antimicrobial-resistant pathogenic bacteria from various domestic animals and their habitats. Antimicrobial-resistant bacteria have been detected in a variety of domestic animals and the environments that are affected by stockbreeding [2,3]. *E. coli*, a type of bacteria common in the intestine of warm-blooded animals, was widely used as an indicator of fecal contamination in drinking water system assessment and food safety evaluation [4]. Pathogenic *E. coli* is an important pathogen that can infect humans and animals. Various pathotypes of *E. coli* can be distinguished by the virulence genes [5]. Infection by pathogenic *E. coli* mainly cause diarrhea in domestic livestock, especially in young animals with clinical syndromes including acute severe watery diarrhea, haemorrhage, and sudden death [6]. Among the identified pathotypes, EPEC (enteropathogenic *E. coli*), ETEC (enterotoxigenic *E. coli*), and STEC (Shiga-like toxin-producing *E. coli*) strains represented three major classes of enteric pathogens leading to diarrhoea in humans and animals [7]. Previous studies showed that feces from wild deer could contaminate surface water that may be used as drinking water for humans and/or domestic animals [8]. Thus, the pathogenic *E. coli* strains from domesticated wild animals (e.g., domesticated wild deer) can also be transmitted to humans [9,10].

As domesticated wild animals, China has a large population of farm-raised sika deer (*Cervus nippon*), mainly distributed in three Northeastern provinces (Liaoning, Jilin and Heilongjiang) with about 80% sika deer production in China [11]. Although the farming pattern of farm-raised sika deer is similar to the domestic ruminants (e.g., cattle, sheep); there are still some uniqueness in its farming production. For example, the mainly purpose of farm-raised sika deer in China is to obtain pilose autler rather than for meat production, and the feeding cycle of farm-raised sika deer is usually longer than two years. Therefore, antimicrobials are not added (or only a small amount of antimicrobials are added) into daily feed, but they are used heavily when disease outbreaks. In comparison, the mainly purpose of domestic ruminants in China are meat production with a feeding cycle of less than one year; excessive antimicrobials are applied in the daily feed for improving growth performance. Compare to wild animals (including wild deer), the diarrheic farm-raised deer accepting heavy amounts of
antimicrobials for disease treatment may be a larger reservoir of potential AMR pathogens. However, to our knowledge, few studies have been focused on AMR pathogens of farm-raised diarrheic sika deer and their potential risk to public health. The objective of this study is to characterize the diversity of serological types, the distribution of antimicrobial-resistant patterns and virulence genes of AMR E. coli in the feces from farm-raised diarrheic sika deer in Northeastern China.

Results and Discussion

Serogroup differences between farm-raised sika deer and other wild animals/domestic ruminants

In this study, 10 isolates with morphology of E. coli were randomly picked up from each deer farm (also from each fecal sample) and subjected to biochemical identification. Among a total of 500 suspect bacterial isolates, 220 of them were identified as typical E. coli strains. One hundred ninety of the obtained E. coli isolates were classified into 28 different types of O serogroups, and the remaining did not belong to any known serogroups (untypable or O-rough) (Figure 1). Around 60% of the identified O serogroups belonged to twelve major groups: O2, O128, O26, O142, O154, O35, O9, O27, O126, O45, O111, and O125, with the former five O serogroups being dominant. Four E. coli isolates were affiliated with serogroup O157, which is known to be associated with life threatening diseases [10].

More than a half of the identified O serogroups in this study have never been reported from wild deer to date, and a few peculiar O serogroups identified here (e.g., O9, O26, O130 and O137) might be associated with human and livestock infections [5,12,13]. Prevalence rate of E. coli O157 from diarrheic farm-raised sika deer was 1.8%, which was significantly higher than the former five O serogroups being dominant. Four E. coli isolates were classified into 28 different types of O serogroups, and the remaining did not belong to any known serogroups (untypable or O-rough) (Figure 1). Around 60% of the identified O serogroups belonged to twelve major groups: O2, O128, O26, O142, O154, O35, O9, O27, O126, O45, O111, and O125, with the former five O serogroups being dominant. Four E. coli isolates were affiliated with serogroup O157, which is known to be associated with life threatening diseases [10].

Figure 1. O serogroups distribution among 220 E. coli isolates from farm-raised sika deer sourced from three Northeastern provinces of China. Note: Others refer to O20, O25, O32, O44 (three isolates of each), and O6, O8, O103 (two isolates of each); ND refers to unknown O serogroups.

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Occurrence of virulence genes and pathogenic E. coli groups in the farm-raised sika deer

The majority of the E. coli isolates in this study carried at least one of the twelve tested virulence genes, some of which occurred in high frequency (Table 1). A total of 74 (33.6%) isolates carried only one virulence gene, whereas 128 (58.2%) isolates carried more than one investigated virulence genes. The genes of astA, eaeA, stx1, stx2, fadA, aidA-1, sfaA, eaeA, estB, fucG, and stx1 were present in 134 (60.9%), 43 (19.5%), 42 (19.1%), 40 (18.2%), 28 (12.7%), 28 (12.7%), 24 (10.9%), 18 (8.2%), and 18 (8.2%) of the E. coli isolates, respectively. The estB and fadA genes were present in less than 2.0% of the E. coli isolates, while the fucC gene was not detected. The pathogenic potential of E. coli can be inferred based on virulence genes [5]. A total of 163 (74.1%) isolates were shown to carry at least one of the seven types of virulence genes (eaeA, fucG, fadA, estB, eaeA, estB) in the study. There were 43 (19.5%), 9 (4.1%), and 33 (15.0%) isolates carrying virulence genes of eaeA (EPEC), stx1 (STEC) and stx2 (STEC), respectively; 9 (4.1%) isolates carried virulence genes of stx1 and stx2 (STEC). A total of 69 isolates (31.4%) carried at least one of the ETEC virulence genes (fucA, sfaA, fadA, eaeA, and eaeA) in the study. The frequency of the LT (elt) and ST (estA/estB) genes was 6.8% in the E. coli isolates. About 26.0% (16 out of 65) and 29.2% (2 out of 69) of the ETEC isolates carried fct and F18 (fadA) colonization antigens, respectively, which were proved to be the most important fimbrial adhesins of ETEC causing livestock diarrhoea [12]. None of the obtained isolates carried eaeA and stx (enterohemorrhage E. coli, EHEC) virulence genes.

The astA gene and typical pathogenic E. coli (EPEC, ETEC, and STEC) from the farm-raised sika deer encoded more frequently than that of wild animals (including wide deer), and even than that of some domestic livestock. The astA gene encodes the toxin EAST1, which is associated with diarrhoea of postweaning pigs [5,17]. Among the E. coli isolates from farm-raised sika deer, a high frequency of 60.9% was observed for the astA gene. The frequency of the gene eaeA (expressing the virulence of EPEC) was 19.5% in the obtained E. coli isolates, in comparison with 1.4% and 0.9% for Danish and Slovakia postweaning diarrhea pigs, respectively [5,12]. Fifty-one isolates (23.2% of total obtained) contained stx1 and/or stx2 genes, in contrast with lower frequencies (16.3% and 10.5%) of stx-gene containing E. coli isolates obtained from wild deer [18,19]. The prevalence rates of the EPEC (20.9%) and STEC (23.2%) strains in this study were much higher than those (1.5% for EPEC and 5.5% for STEC) of wildlife in the south Belgium [7]. ETEC causes travelers diarrhoea by producing different combinations of heat labile (LT) and heat stable (ST) enterotoxins. The prevalence rate of ETEC strains was 31.4% in this study, compared with that of 55.3% and 33.2% from diarrheal enterotoxins. The prevalence rate of ETEC strains was 31.4% in this study, compared with that of 55.3% and 33.2% from diarrheal enterotoxins. The prevalence rate of ETEC strains was 31.4% in this study, compared with that of 55.3% and 33.2% from diarrheal enterotoxins. The prevalence rate of ETEC strains was 31.4% in this study, compared with that of 55.3% and 33.2% from diarrheal enterotoxins.
### Table 1. Distribution of O serogroups, major virulence genes and pathotypes of *E. coli* isolates from farm-raised diarrheic sika deer.

| O serogroups | aidA-1 | eaeA | faeG | fanC | fasA | fedA | astA | elt | estA | estB | stx1 | stx2 | pathotype |
|--------------|--------|------|------|------|------|------|------|-----|------|------|------|------|-----------|
| O2 (19)      | 1      | 19   | –    | –    | –    | –    | 17   | –   | –    | –    | 2    | 2    | EPEC (19)* |
| O5 (5)       | 1      | –    | –    | –    | 1    | 1    | –    | –   | –    | –    | 5    | 6    | STEC (11) |
| O6 (2)       | –      | 1    | –    | 1    | –    | –    | 2    | –   | 2    | –    | –    | –    | STEC (2)  |
| O9 (8)       | –      | –    | 4    | 2    | –    | 4    | 4    | –   | 1    | –    | 3    | –    | EPEC (3)  |
| O20 (3)      | –      | –    | –    | –    | 1    | –    | 1    | 3   | –    | –    | –    | –    | ETEC (3)  |
| O25 (3)      | –      | –    | –    | –    | –    | 3    | 1    | –   | 2    | –    | –    | –    | ETEC (3)  |
| O26 (4)      | 4      | 2    | –    | –    | –    | 10   | –    | –   | –    | –    | 4    | –    | EPEC (4)  |
| O26 (1)      | –      | –    | –    | –    | 1    | –    | –    | –   | –    | –    | –    | –    | ETEC (1)  |
| O26 (11)     | –      | –    | –    | –    | –    | –    | 5    | 6   | –    | –    | –    | –    | ETEC (1)  |
| O27 (4)      | –      | –    | 2    | –    | –    | 3    | 3    | 1   | 3    | –    | –    | –    | ETEC (4)  |
| O27 (3)      | –      | 3    | –    | –    | –    | –    | –    | –   | –    | –    | –    | –    | EPEC (3)  |
| O27 (1)      | –      | –    | –    | –    | –    | 1    | –    | –   | –    | –    | –    | –    | NA (1)*   |
| O32 (2)      | –      | –    | –    | –    | 2    | –    | –    | –   | –    | –    | –    | –    | ETEC (2)  |
| O32 (1)      | –      | –    | –    | –    | –    | –    | –    | –   | –    | –    | –    | –    | NA (1)    |
| O44 (3)      | –      | 3    | –    | –    | –    | –    | 3    | –   | –    | –    | –    | –    | EPEC (3)  |
| O45 (7)      | 6      | –    | –    | –    | –    | 6    | –    | –   | –    | –    | 7    | –    | STEC (7)  |
| O55 (7)      | 4      | –    | –    | –    | –    | 8    | –    | –   | 6    | 1    | –    | –    | STEC (7)  |
| O55 (1)      | 1      | –    | –    | –    | –    | –    | –    | –   | –    | –    | –    | –    | EPEC (1)  |
| O55 (1)      | –      | –    | –    | –    | 1    | –    | –    | –   | –    | –    | –    | –    | ETEC (1)  |
| O77 (5)      | –      | –    | –    | –    | –    | 1    | –    | –   | –    | –    | –    | –    | NA (5)    |
| O86 (4)      | –      | 3    | –    | –    | –    | 3    | –    | –   | –    | –    | –    | –    | EPEC (3)  |
| O87 (1)      | –      | –    | –    | –    | 1    | –    | –    | –   | –    | –    | –    | –    | ETEC (1)  |
| O87 (1)      | –      | 1    | –    | –    | –    | –    | –    | –   | –    | –    | –    | –    | EPEC (1)  |
| O87 (2)      | –      | –    | –    | –    | –    | 2    | –    | –   | –    | –    | –    | –    | NA (2)    |
| O88 (4)      | –      | 2    | –    | 1    | –    | 2    | 4    | –   | 3    | –    | –    | –    | ETEC(4)   |
| O88 (1)      | –      | –    | –    | –    | –    | 1    | –    | –   | –    | –    | –    | –    | NA (1)    |
| O91 (5)      | 2      | –    | –    | –    | –    | 2    | –    | –   | –    | –    | 5    | –    | STEC (5)  |
| O103 (2)     | 1      | –    | –    | –    | –    | 1    | –    | –   | –    | –    | 2    | –    | STEC (2)  |
| O111 (4)     | –      | –    | –    | –    | –    | 4    | –    | –   | –    | –    | 4    | –    | STEC (4)  |
| O111 (1)     | 1      | 1    | –    | –    | –    | –    | –    | –   | –    | –    | –    | –    | EPEC (1)  |
| O111 (1)     | –      | –    | –    | –    | 1    | –    | –    | –   | –    | –    | –    | –    | ETEC (1)  |
| O11 (1)      | –      | –    | –    | –    | –    | 1    | –    | –   | –    | –    | –    | –    | NA (1)    |
| O16 (1)      | –      | –    | –    | –    | 1    | –    | 1    | –   | –    | –    | –    | –    | ETEC (1)  |
| O16 (3)      | –      | –    | –    | –    | –    | 3    | –    | –   | –    | –    | –    | –    | NA (3)    |
| O125 (3)     | –      | –    | –    | –    | 3    | –    | –    | –   | 1    | 3    | –    | –    | STEC (3)  |
| O125 (3)     | –      | –    | –    | –    | 2    | –    | –    | –   | –    | –    | –    | –    | ETEC (2)  |
| O125 (1)     | 1      | –    | –    | –    | –    | 1    | –    | –   | –    | –    | –    | –    | EPEC (1)  |
| O126 (8)     | –      | 4    | –    | 6    | –    | 4    | 2    | 1   | –    | –    | –    | –    | STEC (8)  |
| O128 (7)     | 1      | –    | –    | –    | –    | 7    | –    | –   | 7    | –    | –    | –    | STEC (7)  |
| O128 (2)     | 2      | 2    | –    | –    | –    | –    | –    | –   | –    | –    | –    | –    | EPEC (2)  |
| O128 (8)     | –      | 5    | –    | 4    | –    | 4    | 4    | 2   | 4    | –    | –    | –    | STEC (8)  |
| O128 (1)     | –      | –    | –    | –    | 1    | –    | –    | –   | –    | –    | –    | –    | NA (1)    |
| O138 (2)     | –      | –    | –    | –    | 2    | –    | –    | –   | –    | –    | –    | –    | NA (2)    |
| O138 (3)     | –      | –    | –    | –    | –    | –    | –    | –   | –    | –    | –    | –    | NA (3)    |
| O142 (8)     | 2      | 8    | –    | –    | –    | 1    | –    | –   | –    | –    | –    | –    | EPEC (8)  |
| O142 (1)     | –      | –    | –    | –    | 1    | –    | –    | –   | –    | –    | –    | –    | ETEC (1)  |
| O142 (4)     | –      | –    | –    | –    | –    | 1    | –    | –   | –    | –    | –    | –    | NA (4)    |
| O154 (5)     | –      | –    | –    | –    | 5    | –    | 2    | 1   | –    | –    | –    | –    | ETEC (5)  |
Table 1. Cont.

| O serogroups | aidA-1 | eaeA | faeC | fasA | fedA | astA | elt | estA | estB | stx1 | stx2 | pathotype |
|--------------|--------|------|------|------|------|------|-----|------|------|------|------|-----------|
| O154 (7)     | 2      | 0    | 5    | 0    | 0    | 0    | 0   | 0    | 0    | 0    | 0    | NA (7)    |
| O157 (4)     | 2      | 0    | 5    | 0    | 0    | 0    | 0   | 0    | 0    | 0    | 0    | STEC (4)  |
| O?           | 1      | 0    | 5    | 0    | 0    | 0    | 0   | 0    | 0    | 0    | 0    | STEC (7)  |
| O?           | 2      | 0    | 5    | 0    | 0    | 0    | 0   | 0    | 0    | 0    | 0    | STEC (12) |
| O?           | 1      | 0    | 5    | 0    | 0    | 0    | 0   | 0    | 0    | 0    | 0    | EPEC (3)  |
| O?           | 2      | 0    | 5    | 0    | 0    | 0    | 0   | 0    | 0    | 0    | 0    | NA (8)    |

*Number in bracket represents the total of isolates.

NA represents the isolates not allocated into pathotypes of EPEC, ETEC and STEC.

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antimicrobials. Forty-eight (21.8%) E. coli isolates were capable of resisting 9 antimicrobials. The most frequently resisted antimicrobials were sulfadiazine, sulfanethazine, tetracycline, ampicillin, amoxicillin, chloramphenicol, gentamicin, and ceftriaxone, which were the main antimicrobials used for sika deer diarrhea treatment. These results suggested that the antimicrobials resistance of the E. coli might be derived from the antimicrobials overuse of their hosts.

Similar to the livestock in China, farm-raised sika deers are usually supplied with heavy antimicrobials (e.g., cephalosporin, fluoroquinolone, aminoglycoside and sulfonamides) for disease treatment. Nearly all of the E. coli isolates in this study resisted 4 or more antimicrobials, which was similar to that of food-producing animals reported in China and other countries [1,22,23,24]. Due to less contact with antimicrobials, much lower abundances (7.3% and 8.8%, respectively) of the E. coli isolates from deer in Norway and USA were resistant to one or more of antimicrobials [8,25]. A few previous studies showed that more than 60% of the E. coli isolates from humans and food-producing animals in China were resistant to fluoroquinolone drugs [23,26]. In this study, 34.1%, 36.8%, and 35.0% of the E. coli isolates resisted ciprofloxacin, enrofloxacin, and norfloxacin, respectively. However, fluoroquinolone resistance was relatively low in other countries. For example, only 2.9% of the E. coli isolates from Danish dogs were resistant to ciprofloxacin and 8.0% of the E. coli isolates from Korean pigs were resistant to enrofloxacin [27,28]. The E. coli isolates from wild deer were very sensitive to cephems [7]. Whereas, the cephems resistance of E. coli isolates this study was even much higher (26.4–48.2%) than that of food-producing animals (8.4% and 6.3%) [29,30]. These significant differences may be ascribed to the fact that the antimicrobials are frequently overused on the deer farms in China.

The pathotypes and antimicrobials resistant genotypes of the obtained E. coli isolates resisting tetracycline, sulfonamides, and streptomycin-spectinomycin are shown in Table 3. The genes of aidA, tetA, strA, strB and sul2 were the dominant antimicrobial-resistant genotypes in this study. For the analysis of tetracycline-resistant genes, tetA, tetB and tetC were found in 72, 29, and 10 strains, respectively. In contrast, 86 tetracycline-resistant isolates did not carry any of the tested tetracycline-resistant genes, indicating that other tetracycline-resistant genes (e.g., tetD, tetE or tetM) might be present, or other novel genetic resistant determinants exist [31]. Interestingly, at least one of the tested tetracycline-resistant genes (e.g., tetA, tetB or tetC) was detected in 6 tetracycline susceptible isolates. For the sulfonamides resistance analysis, genes of sul1, sul2, and sul3 were found in 12, 98, and 5 isolates, respectively. Similar to the results of the tetracycline-resistant genes, at least one of the sulfonamides resistant genes (e.g., sul1, sul2 or sul3) was detected in 20 sulfonamides susceptible isolates. For the aminoglycoside resistance, genes of adaA, strA, strB and strC were found in 55, 114, and 123 isolates, respectively. Twenty-five aminoglycoside resistant isolates did not carry any of the tested aminoglycoside resistant genes. However, at least one of the aminoglycoside resistant genes (e.g., strA, strB or adaA) was detected in 73 streptomycin and spectinomycin susceptible isolates.

It is notable that majority (187 of 220) of the obtained E. coli isolates carried at least one of antimicrobial resistant genes that encodes resistant phenotypes to tetracycline, sulfonamides and streptomycin/spectinomycin, corresponding to high abundances of the genes of tetA, sul2 and strA/strB. The tetracycline resistance in E. coli isolates from farm-raised sika deer was mostly due to tetA and tetB, and the frequency of tetA (32.7%) was obviously higher than tetB (13.2%). However, previous studies showed that the frequency of tetB (49.8%) was higher than tetA (24.0%) for the E. coli isolates from pigs raised under overuse of antimicrobials in China [32]. The sulfonamides resistant E. coli is generally attributed to the presence of sul1, sul2 and/or sul3 genes [33]. The sul2 gene displayed much higher frequency (44.5%) than that of the sul1 and sul3 (5.5% and 2.3%, respectively) in the E. coli isolates from this study. Other studies showed that the genes of sul1, sul2 and sul3 showed equal importance for sulfonamides-resistance in E. coli strains from food-producing animals in China [34,35]. Among streptomycin/spectinomycin resistant genes, the strA and strB genes were detected at the highest frequency (51.8% and 55.9%, respectively). One previous study indicated that the strA and strB genes might be present together to make E. coli strains streptomycin resistance [36]. In addition, twelve streptomycin/spectinomycin susceptible E. coli isolates carried adaA, and the findings are consistent with former studies in which a large reservoir of nonintegrated gene cassettes could exist, but might not be expressed in some streptomycin/spectinomycin sensitive E. coli strains [20,36].

Correlations between resistant and virulence genes in the farm-raised sika deer

Significant correlations (P<0.05) were found between a few virulence and resistant genes (Table 4). For example, the correlation coefficients between the resistant gene sul2 and virulence genes of aidA, elt and strC were 0.350, 0.318, and 0.400, respectively. The correlation coefficient was 0.333...
between the resistant gene strA and the virulence gene aidA, and 0.316 between the resistant gene aadA and the virulence gene fedA. Besides of the above genes, no significant correlation was observed between the remaining resistant genes (tetA, tetB, tetC, sul1, sul2 and strB) and the virulence genes (eaeA, fagG, faaA, astA, estA, estB and stx2). Such weak correlations between the selected resistant and virulence genes suggested that the presence of some virulence genes does not necessarily possess resistant characteristics for the *E. coli* of farm-raised sika deer. The results here was inconsistent with traditional view that frequent exposure to heavy antimicro-

bials might drive the distribution, reassortment and co-location of both resistant and virulence genes onto conjugative plasmids or pathogenicity islands in the pathogens, and the antimicrobial-resisting bacteria are more frequent as the pathogens than that of commensal bacteria [20]. Therefore, other factors may responsible for the observed prevalence and the associations of antimicrobial resistant and virulence genes.
Conclusions

Antimicrobials are used heavily for the farm-raised sika deer when disease outbreaks. The results of this study indicated that the pathogenic groups (EPEC, ETEC and STEC) of E. coli strains occurred at much higher frequency than that of wildlife (including wild deer), and even higher than that of livestock in some developed countries. Furthermore, the antimicrobial resistance of the E. coli strains from farm-raised sika deer was also significantly higher than those reported in wild animals and certain livestock in some countries. Other than domestic livestock, antimicrobial-resisting E. coli strains from domesticated wildlife have become a new heavy contamination source, and already posed high potential risks to public health in Northeastern China. Feasible measurements should be taken for prudently antimicrobials use in domesticated wildlife and livestock to prevent the increasingly antimicrobial resistance problem from worsening. In addition, comprehensive surveys on domesticated wildlife for the antimicrobial-resisting bacteria are also strongly recommended to ensure the safety of food products and environments.

Materials and Methods

Ethics Statement

The fecal samples of the present study were collected from sika deer farms, no specific permissions were required for these

Table 2. Antimicrobial resistant phenotypes of E. coli strains isolated from farm-raised sika deer (n = 220).

| Antimicrobials Group | Specific list | MIC(μg/ml) | Number of resistant strains (%) |
|----------------------|--------------|------------|---------------------------------|
|                      |              | Resistance breakpoint | Range | MIC 50% | MIC 90% |
| Amino-glycosides     | Amikacin     | 64 | 1–256 | 16 | 128 | 98 (44.5%) |
|                      | Gentamicin   | 16 | 0.125–512 | 8 | 256 | 124 (56.4%) |
|                      | kanamycin    | 64 | 0.5–128 | 4 | 64 | 44 (20.0%) |
|                      | Streptomycin | 64 | 0.5–512 | 16 | 256 | 88 (40.0%) |
|                      | Spectinomycin| 64 | 0.5–512 | 16 | 128 | 76 (34.5%) |

Cepems (parental)

|                      | Ceftriaxone  | 64 | 1–512 | 16 | 128 | 106 (48.2%) |
|                      | Cefotaxime   | 64 | 1–512 | 16 | 128 | 106 (48.2%) |

Fluoroquinolones

|                      | Ciprofloxacin| 4  | 0.0625–512 | 1 | 32 | 75 (34.1%) |
|                      | Enrofloxacin | 2  | 0.0625–512 | 1 | 32 | 81 (36.8%) |
|                      | Norfloxacin  | 16 | 0.25–512 | 1 | 32 | 77 (35.0%) |

Folate pathway inhibitors

|                      | Sulfadiazine | 512 | 8–512 | >512 | >512 | 197 (89.5%) |
|                      | Sulfamethazine| 512 | 8–512 | >12 | >512 | 182 (82.7%) |

Penicillins

|                      | Amoxicillin  | 32 | 1–512 | 128 | 512 | 149 (67.7%) |
|                      | Ampicillin   | 32 | 1–512 | 128 | 512 | 157 (71.4%) |

Penicol

|                      | Chloramphenicol| 32 | 1–512 | 128 | 512 | 143 (65.0%) |

Tetracycline

|                      | Tetracycline  | 16 | 0.125–512 | 64 | 256 | 176 (80.0%) |

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Table 3. Antimicrobial resistant genotypes and pathotypes of E. coli strains from farm-raised sika deer (n = 220).

| antimicrobials Group | Resistant gene | EPEC | ETEC | STEC | Other | Number of resistant strains |
|----------------------|----------------|------|------|------|-------|-----------------------------|
| Tetracycline         | tetA           | 16   | 22   | 21   | 13    | 72                          |
|                      | tetB           | 9    | 6    | 7    | 7     | 29                          |
|                      | tetC           | 1    | 4    | 4    | 3     | 10                          |
| Sulfonamides         | sulA           | 1    | 5    | 2    | 4     | 12                          |
|                      | sul2           | 23   | 30   | 20   | 25    | 98                          |
|                      | sul3           | 1    | 2    | 1    | 1     | 5                           |
| Streptomycin/spectinomycin | strA | 18   | 37   | 30   | 29    | 114                         |
|                      | strB           | 22   | 41   | 28   | 32    | 123                         |
|                      | aadA           | 17   | 2    | 17   | 19    | 55                          |

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locations/activities, the sika deer farms are the public open place in China, and the sample activities did not involve any endangered or protected species. This study focuses on the microbial antimicrobial-resistant characteristics of fecal samples from farm-raised sika deer, and the vertebrate materials are not included.

Sample collection and *E. coli* strain isolation

*E. coli* isolates of this study were obtained from farm-raised sika deer with clinic signs of yellow/white diarrhea. All fecal samples were collected from 50 sika deer farms located in three Northeastern provinces of China, including 18 farms from Jilin province, 15 farms from Heilongjiang province and 17 farms from Liaoning province during the period of March to October in 2009 (Figure 2). In China, the deer farms are relatively small and usually raise less than 20 sika deers in each farm. One diarrheic fecal sample was collected from one location, and the antimicrobials usage information was obtained from the owners and from the medical records in each farm. The information of the antimicrobials usage in the studied farms within the last 12 months before sampling is shown in Table S1 and Figure S1. The antimicrobials of usage in the studied farms within the last 12 months before medical records in each farm. The information of the antimicrobials were randomly picked and identified by biochemical methods (Gram reaction; activities of catalase, oxidase and urease; indole production, methyl red reaction, Voge-Proskauer test and citrate utilization), and by the API 20E strips (BioMérieux, France) according to the manufacture instructions. The isolates identified as *E. coli* were maintained on Luria-Bertani (LB, Oxoid) slants at 4°C, and as glycerol suspension (20%, v/v) at −80°C for long-term preservation.

### Determination of O serogroups of *E. coli* isolates

Sika deer *E. coli* isolates proliferated on a nutrient agar (NA, BD) were suspended in 0.9% (w/v) NaCl and then autoclaved at 1.05 kg f/cm² for 1 h to extract somatic antigens. The serogroups of the *E. coli* isolates were examined by slide agglutination using O antisera commercially available from China Institute of Veterinary Drugs Control (IVDC, Beijing), and a NaCl control was performed to eliminate false positive results. Positive reactors were confirmed by tube agglutination test [38].

### Antimicrobial susceptibility test

Minimal inhibitory concentration (MIC) determination was performed by using the broth micro-dilution method according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) Guideline (CLSI, 2009). The following 16 antimicrobials were selected for susceptibility test: amikacin, gentamicin, kanamycin, streptomycin, spectinomycin, ceftiofur, ceftriaxone, ciprofloxacin, enrofloxacin, norfloxacin, sulfadiazine, sulfamethazine, amoxicillin, ampicillin, chloramphenicol and tetracycline. The breakpoints for each antimicrobial resistance were outlined in Table 2 according to the CLSI guidelines (CLSI, 2009). The isolates showing resistance to one or more antimicrobials were characterized for antibiotic resistant genes. Strain *E. coli* ATCC...
Table 5. Primers and the predicted size of the virulence genes associated with different E. coli pathotypes.

| Virulence factors | E. coli pathotypes | Description/function | Primers | Primer sequences | predicted sizes | Reference |
|-------------------|--------------------|----------------------|---------|-----------------|----------------|----------|
| LT (elt)          | ETEC               | Heat-labile toxin    | elt-F   | GCC GTT ACT ATC | 272            | [39]     |
|                   |                    |                      | elt-R   | TGG TCT CGG TCA |                |          |
| STa (estA)        | ETEC               | Heat-stable enterotoxin a | estA-F | CAA CTG AAA TCA | 158            | [39]     |
|                   |                    |                      | estA-R  | TTA ATA ACA TCC |                |          |
| STb (estB)        | ETEC               | Heat-stable enterotoxin b | estB-F | CGC TGA ATG TCA | 113            | [39]     |
|                   |                    |                      | estB-R  | CGT GGT ATA GTC |                |          |
| Stx1 (stx1)       | STEC (EHEC)        | Shiga toxin I        | stx1-F  | CGC TGA ATG TCA | 302            | [40]     |
|                   |                    |                      | stx1-R  | CGT GGT ATA GTC |                |          |
| Stx2 (stx2)       | STEC (EHEC)        | Shiga toxin II       | stx2-F  | CTT CGG TAT CCT | 516            | [40]     |
|                   |                    |                      | stx2-R  | CTG TGA CAG TGA |                |          |
| EAST1 (astA)      | EaggEC             | EaggEC (heat-stable enterotoxin) | astA-F | TGAT GCC ATC | 125            | [41]     |
|                   |                    |                      | astA-R  | GCC GGT AGC |                |          |
| F4 (faeG)         | ETEC               | Fimbrial adhesin     | faeG-F   | GAA TCT GTC CGA | 499            | [39]     |
|                   |                    |                      | faeG-R   | GTG GCC ATC CGT |                |          |
| F5 (fanC)         | ETEC               | Fimbrial adhesin     | fanC-F   | TGC GAC TACCAA | 450            | [42]     |
|                   |                    |                      | fanC-R   | TAT CCA CCA TTA |                |          |
| F6 (fasA)         | ETEC               | Fimbrial adhesin     | fasA-F   | TCT GCT CTA AAA | 333            | [39]     |
|                   |                    |                      | fasA-R   | AAC TCC ACC GTT |                |          |
| F18 (fedA)        | ETEC               | Fimbrial adhesin     | fedA-F   | TGG TAA CGT CGT | 313            | [39]     |
|                   |                    |                      | fedA-R   | ACT TAC AGT CGT |                |          |
| AIDA (aidA-1)     | EPEC/DAEC          | Adhesin involved in diffuse adherence | aidA-F | ACA GTA TCA TAT | 585            | [41]     |
|                   |                    |                      | aidA-R   | TGG GCC CGA CAA |                |          |
| EaeA (eaeA)       | EPEC/EHEC          | Intimin              | eae-F    | GGA AGC GCA GCA | 775            | [40]     |
|                   |                    |                      | eae-R    | GCC GCT CAT CAT |                |          |

Note: DAEC refers to diffusely adherent E. coli; EaggEC refers to Enteroaggregative E. coli; EHEC refers to Enterohemorrhage E. coli.

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25922 was applied as quality control for the susceptibility testing procedure.

Polymerase chain reaction (PCR) detection of virulence genes in E. coli isolates

The PCR was applied to detect whether the obtained E. coli isolates harboring toxins (LT, STa, STb, Stx1, Stx2, and EAST1) and adhesions (F4, F5, F6, F18, AIDA, and EaeA), known as virulence genes for E. coli pathotypes causing intestinal diseases in humans and animals. The information of specific oligonucleotide primers, amplicons predicted sizes and pathotypes definition (EPEC, STEC, ETEC and EHEC) for the tested virulence genes are shown in Table 5. The PCR conditions for each virulence gene were performed as described previously [39,40,41,42]. Three strains from Zhejiang Province Key Laboratory for Food Safety were selected as positive controls for determining pathotypes of EPEC, ETEC and STEC.

Antimicrobial-resisting gene detection

Antimicrobial-resisting genes were detected and identified following the protocols as described previously (Table 6). Briefly, the E. coli strains were grown in 500 µL LB broth overnight, and 20 µl of the culture was transferred to 200 µl lysis buffer [0.1 M Tris-HCl (pH 8.5), 0.05% Tween 20, and 0.24 mg/ml proteinase K]. The sample was incubated at 60°C for 1 hour and subsequently heated at 97°C for 15 min. The PCR primers and annealing temperatures for major resisting genes of tetracycline (tetA, tetB and tetC), sulfonamides (sul1, sul2 and sul3), and streptomycin-spectinomycin (strA/strB and aadA) were detailed in Table 6, and the major resisting genes were amplified by a set of multiplex PCR protocols [36,43,44]. The multiplex PCRs were all performed with a total 25-µl reaction mixture and a Qiagen multiplex PCR kit (Qiagen, Shanghai) with 1 µl Qiagen multiplex PCR master mixture, 1× Q-solution, and 1× primer mixture according to the manufacturer’s instructions. The PCRs were performed as follows: 1 cycle of 4 min at 95°C; 35 cycles, each consisting of 1 min at 95°C, 1 min at annealing temperature, and 1 min at 72°C; and 1 cycle of 7 min at 72°C.

Statistical analysis

For the purpose of statistical analysis, isolates with reduced susceptibility were classified into resistant groups. The pairwise statistical associations between major antimicrobial-resisting and virulence genes were determined by using the Statistical Package...
for the Social Sciences (SPSS version 13.0; SPSS, Chicago, IL, USA).

Supporting Information

Figure S1 Frequency of antimicrobials usage in 50 sika deer farms. Note: Antimicrobials abbreviations are the same as Table S1.

Table S1 Antimicrobials usage information in 50 sample locations. Antimicrobials abbreviations: AMI, Amikacin; EN, Gentamicin; KAN, Kanamycin; STR, Streptomycin; SPE, Spectinomycin; CER, Ceftiofur; CEE, Ceftriaxone; CIP, Ciprofloxacin; ENR, Enrofloxacin; NOR, Norfloxacin; SDM, Sulfadiazine; SMZ, Sulfamethazine; AMO, Amoxicillin; AMP, Ampicillin; CHL, Chloramphenicol; TET, Tetracycline; Note: , the antimicrobial was used; #, the antimicrobial was not used. Note: Sample locations of LS, JS and HS are the same as Figure 2.

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Author Contributions

Conceived and designed the experiments: YZ HJ.Performed the experiments: RL LH LH. Analyzed the data: YZ QW. Contributed reagents/materials/analysis tools: RL LH LH. Wrote the paper: RL YZ HJ.

References

1. Lei T, Tian W, He L, Huang XH, Sun YX, et al. (2010) Antimicrobial resistance in Escherichia coli isolates from food animals, animal food products and companion animals in China. Vet Microbiol 146: 85–89.
2. Chen B, Zheng W, Yu Y, Huang W, Zheng S, et al. (2011) Class 1 integrons, selected virulence genes, and antibiotic resistance in Escherichia coli isolates from the Minjiang River, Fujian Province, China. Appl Environ Microbiol 77: 148–155.
3. Sanchez S, Martinez R, Garcia A, Blanco J, Blanco JE, et al. (2009) Longitudinal study of Shiga toxin-producing Escherichia coli shedding in sheep flocks: persistence of specific clones in sheep flocks. Appl Environ Microbiol 75: 1769–1775.
4. Schets FM, van Wijnen JH, Schijven JF, Schoon H, de Roda Husman AM (2008) Monitoring of waterborne pathogens in surface waters in amsterdam, the Netherlands, and the potential health risk associated with exposure to cryptosporidium and giardia in these waters. Appl Environ Microbiol 74: 2069–2078.
5. Vu Khe H, Holoda E, Pilepichez E, Blanco M, Blanco JE, et al. (2006) Serotypes, virulence genes, and PFGE profiles of Escherichia coli isolated from pigs with postweaning diarrhea in Slovakia. BMC Vet Res 2: 10.
6. Mackintosh C, Haigh JC, Griffin F (2002) Bacterial diseases of farmed deer and bunos. Rev Sci Tech 21: 249–263.
7. Bardao M, Gregoire F, Mayoret A, Nahayo A, Duprez JN, et al. (2010) Enteropathogenic (EPEC), enterohaemorragic (EHEC) and verotoxigenic (VTEC) Escherichia coli in wild cervids. J Appl Microbiol 109: 2214–2222.
8. Lillegaard A, Bergan B, Schau J, Bruheim T, Vikoren T, et al. (2005) Campylobacter spp., Salmonella spp., verocytotoxic Escherichia coli, and antibiotic resistance in indicator organisms in wild cervids. Acta Vet Scand 46: 23–32.
9. Nagono H, Hirochi T, Fujita K, Wakamori Y, Takeshi K, et al. (2004) Phenotypic and genotypic characterization of beta-D-glucuronidase-positive Shiga toxin-producing Escherichia coli O157: H7 isolates from deer. J Med Microbiol 53: 1037–1043.
10. Rabatsky-Ehr T, Dingman D, Marcus R, Howard R, Kinney A, et al. (2002) Deer meat as the source for a sporadic case of Escherichia coli O157: H7 infection, Connecticut. Emerg Infect Dis 8: 525–527.
11. Dale R, McCullough ST, Krichi Kaji (2009) Biology and Management of Native and Introduced Populations. New York: Springer Tokyo Berlin Heidelberg: 521–539.

Table 6. Primers and single PCR conditions of the 9 resistant genes.

|Gene  | Primer name | Oligonucleotide sequences of primers | Annealing (°C) | Amplified Products (bp) | Reference |
|------|-------------|-------------------------------------|----------------|------------------------|-----------|
|tetA | tetA-F      | GGC GGT CTT CTT CAT CAT GC          | 64             | 502                    | [36]      |
|      | tetA-R      | CGG GCA GCA GAA GAA GTA GA          |                |                        |           |
|tetB | tetB-F      | CAT TAA TAG GGC CAT GGC TG          | 64             | 930                    | [36]      |
|      | tetB-R      | TGA AGG TCA TCG ATG GCA TG          |                |                        |           |
|tetC | tetC-F      | GCT GTA GGC ATA GGC TTG GT          | 64             | 888                    | [36]      |
|      | tetC-R      | GCC GGA AGC GAG AAG AAT CA          |                |                        |           |
|sul1 | sul1-F      | GTG AGG GTG TTC GGC ATT CT          | 68             | 779                    | [36]      |
|      | sul1-R      | TCC GAG AAT GTG ATT GCG CT          |                |                        |           |
|sul2 | sul2-F      | CGG CAT CGT CAA CAT AAC CT          | 66             | 721                    | [36]      |
|      | sul2-R      | TGT GCG GAT GAA GTC AGC TC          |                |                        |           |
|sul3 | sul3-F      | GAG CAA GAT TTT TGG AAT CG          | 51             | 880                    | [43]      |
|      | sul3-R      | CAT CTG CAG CTA ACC TAG GGC TTT GGA|                |                        |           |
|strA | strA-F      | CTT GGT GAC GGC AAC ATC             | 55             | 546                    | [44]      |
|      | strA-R      | CCA ATC GCA GAT AGA AGC C           |                |                        |           |
|strB | strB-F      | ATC GTC AAG GGA TTG AAA CC          | 55             | 509                    | [44]      |
|      | strB-R      | GGA TCG TAG AAC ATA TTG GC          |                |                        |           |
|aadA | aadA-F      | GTG GAT GGC GCC CTG AAG CC          | 68             | 525                    | [44]      |
|      | aadA-R      | AAT GCC CAG TCG GCA GCG             |                |                        |           |
12. Frydenhal K (2002) Prevalence of serogroups and virulence genes in Escherichia coli associated with postweaning diarrhoea and edema disease in pigs and a comparison of diagnostic approaches. Vet Microbiol 83: 169–182.

13. Yamamoto T, Nakazawa M (1997) Detection and sequences of the enterosaggregative Escherichia coli heat-stable enterotoxin 1 gene in enterotoxigenic E. coli strains isolated from piglets and calves with diarrhea. J Clin Microbiol 35: 223–227.

14. Dunn JR, Keen JE, Moreland D, Alex T (2004) Prevalence of Escherichia coli O157:H7 in white-tailed deer from Louisiana. J Wildl Dis 40: 361–365.

15. Renter DG, Sargeant JM, Hygstrom SE, Hoffman JD, Gillespie JR (2001) Escherichia coli O157:H7 in free-ranging deer in Nebraska. J Wildl Dis 37: 755–760.

16. R.G VAAaJ (2010) Prevalence and Comparative Studies of Some Major Serotype of E. coli from Cattle and Buffalo Calf Scour. Veterinary World, 3: 438–459.

17. Osek J (2003) Detection of the enterosaggregative Escherichia coli heat-stable enterotoxin 1 (EAST1) gene and its relationship with fimbral and enterotoxin markers in E. coli isolates from pigs with diarrhea. Vet Microbiol 91: 65–72.

18. Asakura H, Makino S, Shirahata T, Tsukamoto T, Kurazono H, et al. (1998) Detection and genetical characterization of Shiga toxin-producing Escherichia coli from wild deer. Microb Immunol 42: 815–822.

19. Fukayama M, Ysokoyama R, Sakata S, Furuhata K, Oonaka K, et al. (1999) Study on the verotoxin-producing Escherichia coli-isolation of the bacteria from deer dung. Kansenshogaku Zasshi 73: 1140–1144.

20. Boerlin P, Travis R, Gyles CL, Reid-Smith R, Janecko N, et al. (2005) Patterns of antimicrobial resistance observed in Escherichia coli isolated from pigs, cattle and sheep in Canada. J Vet Microbiol 75: 73–82.

21. Myers LL, Guinee PA (1976) Occurrence and characteristics of enterotoxigenic Escherichia coli in swine. J Clin Microbiol 42: 3483–3489.

22. Asai T, Kojima A, Harada K, Ishihara K, Takahashi T, et al. (2005) Correlation of multiple-antimicrobial-resistant Escherichia coli isolates from diseased chickens and swine in China. J Clin Microbiol 42: 3483–3489.

23. Dai L, Lu LM, Wu CM, Li BB, Huang SY, et al. (2008) Characterization of antimicrobial resistance determinants in avian Escherichia coli isolates from China. J Antimicrob Chemother 60: 775–781.

24. Ojima J, Ueno Y, Kimura K, Yamanaka M, Sawada K, et al. (2005) Detection of virulence genes and occurrence of antimicrobial resistance in Escherichia coli isolated from humans and food-producing animals. Lett Appl Microbiol 49: 627–634.

25. Sayah RS, Kaneene JB, Johnson Y, Miller R (2005) Prevalence of antimicrobial resistant enteric pathogens of food animals in North America. Zentralbl Veterinarmed B 52: 49–59.

26. Lanz R, Kuhnert P, Boerlin P (2003) Antimicrobial resistance and resistance gene determinants in clinical Escherichia coli from different animal species in Switzerland. Vet Microbiol 91: 73–84.

27. Guinee PA JW, Wadstron T, Sellwood R (1981) Escherichia coli associated with neonatal diarrhea in piglets and calves. Curr Top Vet Anim Sci 13: 126–162.

28. Blaschke BD, Ahrens P, Meyling A (1994) Detection of fimbrial and toxin genes in Escherichia coli isolated from swine. Vet Microbiol 45: 458–459.

29. Liu JH, Wei SY, Ma JY, Zeng ZL, Lu DH, et al. (2007) Detection and characterisation of CTX-M and CMY-2 beta-lactamases among Escherichia coli isolates from farm animals in Guangdong Province of China. Int J Antimicrob Agents 29: 576–581.

30. Yang H, Chen S, White DG, Zhao S, McDermott P, et al. (2004) Characterization of multiple-antimicrobial-resistant Escherichia coli isolates from diseased chickens and swine in China. J Clin Microbiol 42: 3483–3489.

31. Tuckman M, Petersen PJ, Howse AY, Orlowski M, Mullen S, et al. (2007) Occurrence of tetracycline resistance genes among Escherichia coli isolates from the phase 3 clinical trials for tigecycline. Antimicrob Agents Chemother 51: 3205–3211.

32. Tang X, Tian C, Zhang X, Zhao Z, Xia X, et al. (2011) Antimicrobial resistances of extraintestinal pathogenic Escherichia coli isolates from swine in China. Microb Pathog 50: 207–212.

33. Hammerum AM, Sandbang D, Andersen SR, Seyfarth AM, Pardo LS, et al. (2006) Detection of sul2 and sul3 in sulphonamide resistant Escherichia coli isolates obtained from healthy humans, pork and pigs in Denmark. Int J Food Microbiol 106: 233–237.

34. Ho PL, Wong RC, Chow KH, Que TL (2009) Distribution of integron-associated trimethoprim-sulphonamide resistance determinants among Escherichia coli from humans and food-producing animals. Lett Appl Microbiol 49: 627–634.

35. Zhang T, Wang CG, Zhong XH (2012) Survey on sulphonamide antibiotic resistance genotype and phenotype of avian Escherichia coli in North China. Poult Sci 91: 884–887.

36. Guinee PA, Wadstron T, Sellwood R (1981) Escherichia coli associated with neonatal diarrhea in piglets and calves. Curr Top Vet Anim Sci 13: 126–162.

37. Ngeleka M, Pritchard J, Appleyard G, Middleton DM, Fairbrother JM (2003) Identification of toxin and pilus genes in porcine Escherichia coli using polymerase chain reaction (PCR) with multiple primer pairs. Gen Meet Am Soc B, 509.

38. Blanco M, Blanco JE, Mora A, Dahbi G, Alonso MP, et al. (2004) Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing Escherichia coli isolates from cattle in Spain and identification of a new intimin variant gene (eae-xi). J Clin Microbiol 42: 645–651.

39. Ngelale M, Pritchard J, Appleyard G, Middelton JM, Fairbrother JM (2003) Isolation and association of Escherichia coli AIDA-I/STb, rather than EAST1 pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates. J Vet Diagn Invest 15: 242–252.

40. Ojeniyi B, Ahrens P, Meyling A (1994) Detection of intimin 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. Zentralbl Veterinarmed B 41: 49–59.

41. Perreten V, Boerlin P (2003) A new sulfonamide resistance gene (sul3) in Escherichia coli isolated from swine in the United States. Zentralbl Veterinarmed B 41: 49–59.

42. Boerlin P, Renter DG, Sargeant JM, Hygstrom SE, Hoffman JD, Gillespie JR (2001) Prevalence and Comparative Studies of Some Major Serotype of E. coli from Cattle and Buffalo Calf Scour. Veterinary World, 3: 438–459.

43. Perreten V, Boerlin P (2003) A new sulfonamide resistance gene (sul3) in Escherichia coli isolated from swine in the United States. Zentralbl Veterinarmed B 41: 49–59.

44. Hammerum AM, Sandbang D, Andersen SR, Seyfarth AM, Pardo LS, et al. (2006) Detection of sul2 and sul3 in sulphonamide resistant Escherichia coli isolates obtained from healthy humans, pork and pigs in Denmark. Int J Food Microbiol 106: 233–237.

45. Ho PL, Wong RC, Chow KH, Que TL (2009) Distribution of integron-associated trimethoprim-sulphonamide resistance determinants among Escherichia coli from humans and food-producing animals. Lett Appl Microbiol 49: 627–634.

46. Blaschke BD, Ahrens P, Meyling A (1994) Detection of intimin 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. Zentralbl Veterinarmed B 41: 49–59.

47. Perreten V, Boerlin P (2003) A new sulfonamide resistance gene (sul3) in Escherichia coli isolated from swine in the United States. Zentralbl Veterinarmed B 41: 49–59.

48. Madsen L, Aarestrup FM, Olsen JE (2000) Characterisation of streptomycin resistance observed in Escherichia coli isolated from domestic deer. Zentralbl Veterinarmed B 47: 1094–1099.