Virulence genes of Escherichia coli (E. coli) isolates from healthy dairy cows were identified and characterized by a multiplex PCR assay and serogrouping test. The results showed that among the target genes, eaeA was most frequently detected, accounting for 22.11% (67/303) in all strains from 101 cows. For categorization of E. coli, aEPEC was the category with widest distribution detected in 55 (18.15%) strains from 22 cattle. All of 84 PCR-positive strains belonged to 14 O serogroups, and O149 (25.00%) was most common identified, followed by O2 (17.86%), O8 (11.90%) and O103 (9.52%) with relatively high prevalence.

**Key words:** E. coli; Virulence gene; Multiplex PCR; Serogroup; Healthy dairy cow

**INTRODUCTION**

E. coli is an opportunistic species comprising the normal flora in the gastrointestinal tracts of the warm-blooded animals. It is usually non-pathogenic kept in host intestines. However, in the immunocompromised or intestinal flora disturbed hosts, or when the E. coli in the host intestines become translocated, normal E. coli strains can cause relevant diseases as a pathogenic one (1).

Pathogenic E. coli is increasingly isolated from cases of human and animal diseases (2, 3). Moreover, it has also been detected from healthy humans and animals. In 2001, Orden et al. (2001) (4) reported some causative agents (cytotoxic necrotizing factors, verotoxins and eae gene) of pathogenic E. coli in healthy cows, sheep and goats. Amar et al. (2007) (5) reported existence of enteroaggregative E. coli in asymptomatic individuals in England. Some researches in other countries also isolated pathogenic E. coli from healthy animals (6, 7). However, very few studies have examined the pathotypes of E. coli in healthy animals in China. In present study, the virulence genes of E. coli were investigated and characterized to elucidate the prevalence of pathogenic E. coli in healthy cattle.

PCR (Polymerase chain reaction) method, particularly multiplex PCR, being a rapid and effective gene detection assay, is increasingly popular in identification of various bacteria (8, 9). Some multiplex PCRs have been reported elsewhere to detect virulence genes of E. coli. Bai et al. (2010)
Huasai, S. et al. Virulence genes of E. coli

(10) reported a multiplex PCR procedure that detected fliC, stx1, stx2, eae, rfbE, and hlyA genes, which are the major virulence genes of E. coli O157:H7. In the study conducted by Kwai and Ke (2009) (11), a multiplex PCR assay was applied to a panel of 87 E. coli isolates from different sources to simultaneously detect virulence genes, such as stl, lt1, lt2, vt1, vt2 and eaeA. Moreover, some researches focused on the detection of virulence genes in combination with serogroup-specific genes to test serogroups of E. coli using multiplex PCR methods (12, 13). We can effectively save time and effort involved in the determination of various virulence genes through multiplex PCR technique.

MATERIALS AND METHODS

E. coli strains and control strains

Forty unrelated family dairy farms in Hulunbeier area (Inner Mongolia, Northern China), were randomly selected for test. The population in this area is predominantly engaged in animal husbandry. Stool specimens were collected from the rectums of 212 apparently healthy dairy cows (20% of the herd or a minimum of 5 cows from each farm). The specimens were transported on ice to the laboratory. Swabs of stool were inoculated onto the surface of eosin–methylene blue agars (EMB) and streaked for isolated colonies. After incubation for 24 h at 37 °C, three or four colonies with typical E. coli morphology and one of another morphological type were streaked on fresh plates. Each colony was independently subjected to Gram staining, microscopic examination, biochemical test and serogrouping. Eventually, 303 E. coli strains from 101 cows (three strains for one cow), were randomly selected for characterizing virulence genes. E. coli strains used as controls were C83922 (O101:K99, F41; st+), C83902 (O8:K87, K88ac; st+ lt+), E. coli O157 (stxl stx2+), E. coli O139 (eae+) and E. coli O127 (eaeA+ bfpA+). All strains were stored at -20 °C in LB broth with 10% glycerol.

PCR protocols

Bacterial DNA used for PCR analyses was prepared with a genomic DNA purification kit (Wizard® Genomic DNA Purification kit, Promega Corporation, USA) used according to the manufacturer’s recommendations. Alternatively, bacterial DNA was extracted by a boiling procedure. Single colonies were cultured overnight at 37°C in 1.5 ml tubes containing 1 ml LB broth. Each bacterial suspension was centrifuged for 15 min at 12,000 rpm, and the pellet was resuspended in 200μl double distilled water. After 10 min of boiling in a water bath and centrifugation for 15 min at 12,000 rpm, the supernatant was used as template DNA for PCR assays.

All strains were evaluated by multiplex PCR for identification of six virulence genes. The PCR primers were presented in Table 1. We consulted the references about the conditions for DNA template amplification and further optimized for present multiplex PCR procedures.

Table 1. PCR markers for detection of virulence gene of E. coli

| Target gene | Nucleotide sequence (5’→3’) | Size of amplified product (bp) | References |
|-------------|----------------------------|-------------------------------|------------|
| eaeA        | F: GTGGCGAATACGTGGCGAGACT R: CCCCATATTTTCCACCTGTCG | 891 | 14 |
| bfpA        | F: AATGGGTCTTGGCTGTGGTGTC R: GCGGCTTTATCCTCAAGGTA | 326 | 14 |
| stl         | F: AAATGCGCATCTTGCTGACTGTC R: CAGTCGTCACGTGTCCTG | 370 | 14 |
| stx2        | F: TGCCATTCTGGGCTGGCATGTC R: GGATCTTCTGTCACTGACAC | 283 | 14 |
| st          | F: ATTTTTCTTCTGTATGTGTTCT R: CACCCGTACAAAGCAGATT | 190 | 15 |
| lt          | F: GCC GAC AGA TTA TAC CGT GC R: CGG TCT CTA TAT TCC CTG TT | 450 | 15 |
Bacterial DNA was amplified in the PCR reaction mixtures as follows. Each reaction system had a final volume of 50μl and contained 2.0μl of 10×E, Taq buffer, 4μl of 25 mM MgCl₂, 4μl of dNTPs (each 2.5 mM), 1.25U of E, Taq DNA polymerase, 20-40μM of each primer, and 2μl of the DNA template. All reagents were the products of TaKaRa Biotechnology (Dalian) Co., Ltd, China. The mixtures were preheated at 94°C for 1 min before submitted to recycling step. The amplification conditions for the multiplex PCR assays were 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. A final extension step was performed at 72°C for 10 min. The PCR products were kept at 4°C until removed and were separated in agarose gel (2% w/v) electrophoresis and visualized under UV light after staining with a nucleic acid stain (GoldView™, SBS Genetech, Beijing, China).

Serogrouping

Serological typing of all PCR-positive strains was carried out by slide agglutination with E. coli O antisera (O1-O160). The antisera were obtained from the China Institute of Veterinary Drug Control (Beijing, China).

RESULTS

Of 101 healthy dairy cows, 34 (33.66%) carried at least one virulence gene of E. coli, and of 303 E. coli strains from all animals (three strains were tested for one animal), 84 (27.72%) were positive for the virulence genes (Table 2, seen in page 16). Among the six virulence genes identified, eaeA was the most common gene harbored by 67 (22.11%) strains from 27 cattle. The prevalence of the remaining genes was that bfpA and st were both recovered in 10 (3.30%) strains from four different cattle, lt and stx1 were in seven (2.31%) strains from three cattle respectively and six (0.94%) strains from three cattle, and none of the strains carried the gene for stx2 (Fig. 1).

Table 2. Distribution of virulence genes of E. coli isolates

| Serogroup | No. of Animals | No. of Strains* | Virulence gene of E. coli |
|-----------|----------------|----------------|--------------------------|
|           | eaeA | bfpA | stx1 | stx2 | st | lt |
| O2        | 6    | 15   | +    | -    | -   | -  |
| O8        | 3    | 7    | +    | -    | -   | -  |
| O8        | 1    | 3    | -    | -    | -   | +  |
| O10       | 2    | 5    | +    | -    | -   | -  |
| O44       | 1    | 2    | -    | +    | -   | -  |
| O55       | 1    | 3    | +    | +    | -   | -  |
| O78       | 1    | 3    | -    | -    | -   | +  |
| O78       | 1    | 3    | -    | -    | -   | +  |
| O86       | 1    | 2    | -    | +    | -   | -  |
| O86       | 1    | 3    | +    | -    | -   | -  |
| O103      | 2    | 5    | +    | +    | -   | -  |
| O103      | 1    | 3    | +    | -    | -   | -  |
| O125      | 1    | 2    | -    | -    | +   | -  |
| O136      | 1    | 3    | -    | -    | -   | +  |
| O145      | 1    | 2    | +    | -    | +   | -  |
| O149      | 8    | 21   | +    | -    | -   | -  |
| O152      | 1    | 1    | +    | -    | -   | -  |
| O159      | 1    | 1    | -    | -    | -   | +  |

*: Three E. coli strains were tested for one animal.
E. coli isolates were categorized into enteropathogenic E. coli (EPEC), Shiga-toxin producing E. coli (STEC) and enterotoxigenic E. coli (ETEC), and EPEC was further divided into typical EPEC (tEPEC) and atypical EPEC (aEPEC) according to the related virulence genes detected by the multiplex PCR assays. The distribution of the categories according to priority was as follows: aEPEC detected in 55 (18.15%) strains from 22 cattle, ETEC in 13 (4.29%) strains from five cattle, tEPEC in eight (2.64%) strains from three cattle, and STEC in six (1.98%) strains from three cattle (Fig. 2). An example of the multiplex PCR products for representative isolates is shown in Fig. 3.

Based on the serogrouping test, all of 84 PCR-positive strains covered 14 O serogroups (Table 2, seen in page 16). As shown in the table, O149 (25.00%) was the most common serogroup, followed by O2 (17.86%), O8 (11.90%), O103 (9.52%), O78 (7.14%), O10 (5.95%), O86 (5.95%), O55 (3.57%), O136 (3.57%), O44 (2.38%), O125 (2.38%), O145 (2.38%), O152 (1.19%), and O159 (1.19%).

**DISCUSSION**

Cattle have been considered as important asymptomatic carriers of potential risk factors for diseases of humans and animals. The present study has shown that key virulence genes of pathogenic E. coli could be identified in fecal E. coli isolates from healthy cattle in northern China. The virulence genes examined in this article were chosen because of their association with E. coli strains causing animal and human infections. Our findings showed that eaeA is the most common gene identified in 22.11% (67/303) strains, whereas the frequency of the other test genes is generally less than 4%
Moreover, in the 67 eaeA-positive strains, only 12 (17.91%) were found the combination of eaeA with other genes; the rest 55 strains were recovered eaeA alone (Table 2, seen in page 16). The above results were the main reason for why aEPEC was the most frequent one among the four categories tested. In our study, E. coli strains possessed A/E genotype (eaeA+) or EAF plasmid (bfpA+) were classified as atypical EPEC (aEPEC), while the strains with both of them were as typical EPEC (tEPEC) regardless of their serogroups. For the other categories, the strains with and without the eaeA genotype that harbored Shiga toxin genes (stx1 and/or stx2) were classified as STEC; the strains harbored enterotoxins (st and/or lt) were as ETEC. The aEPEC is increasingly isolated from human and different animal species, and domestic animals have been seen as important reservoir of aEPEC. Krause et al. (2005) (16) reported prevalence of aEPEC as high as 92.8% in attaching and effacing Escherichia coli (AEEC) strains (equivalent to eae-positive E. coli strains in current article) from healthy animals when testing seven different species of domestic animals. Yuste et al. (2008) (17) described 166 of 185 AEEC strains that isolated from diarrheic calves, lambs, and goat kids and from healthy cattle, sheep, and goats were aEPEC. The results of these reports also showed that aEPEC strains were more prevalent than tEPEC strains; this is consistent with our results. In present test, the percentage of aEPEC was significantly higher than tEPEC in AEEC when accounting for 82.09% (55/67), whereas the latter was only found in eight strains. In addition, we found that aEPEC was most frequently detected in all PCR positive strains compared to other categories. The findings of our research indicated that healthy dairy cows were a natural reservoir of aEPEC. STEC is an important pathogen recovered from different clinical isolates and even associated with hemolytic uremic syndrome (HUS) (1), and STEC strains of bovine origin have been detected elsewhere (6, 18). Rigobelo et al. (2006) (18) reported different countries had shown that 10-80% of cattle might carry STEC. However, in our study, a lower rate (1.98%) of STEC isolation was found, and among the identified six STEC strains, four carried eaeA and stx1, two carried stx1 alone, while none of the strains carried stx2. Similar results were described by some other reports in which stx1 genes were more prevalent in bovine STEC isolates than stx2 genes (7, 19). ETEC strains are the major cause of diarrhea in human and in some animal species, especially in neonates (20, 21). ETEC causes diarrhea by producing enterotoxins heat-labile (lt), heat-stable (st) or both (18). The two toxins were frequently found in diarrheal cases of humans, cattle and pigs, sometimes in healthy humans and pigs from different countries (3, 18, 22, 23). However, the distribution of ETEC in healthy cattle has been less noticed. As far as we know, Houser et al. (2008) (24) recently reported O-groups associated with ETEC in healthy lactating dairy cows of a Pennsylvania dairy herd when investigating phenotypic and genotypic diversity of E. coli in 10 cows. Blanco et al. (25) reported the prevalence of ETEC in 1% calves with diarrhea and 4% healthy controls. In present study, 6.93% (7/101) cattle were confirmed to carry ETEC and 13 strains were positive for st and/or lt. The gene for st was detected from 10 strains and for lt was from seven strains, four of them were positive for both, it seems that st was more prevalent. Similar result was described by Rigobelo et al. (2006) (18) in Brazil, for fecal samples from diarrheal dairy calves. A wide range of serogroups in E. coli strains of bovine origin has already been reported either in disease cases or in healthy cattle, when some of them were only found in cattle and some were associated with humans (26). Among the serogroups detected in this survey, O2, O8, O103 and O136 belong to the prevalent serogroups in healthy cattle. For the rest serogroups, O10, O55, O125, O145, O149, and O159 were also identified from healthy cattle whereas with low proportions, especially, O55 was found only in one Japanese healthy cow (27). On the contrary, in our research, O149 was most frequent accounting for 25% (21/84) and O55 was detected in 3.57% (3/84) strains. In addition, we identified four
serogroups (O44, O78, O86, and O152) not reported in healthy cattle strains so far. Three of them have been reported in association with several diseases in human and cattle, namely, O44, O78 and O86 were detected from diarrheic patients (28), O78 was from septicemic calves (29), and O86 was from diarrheic calves (18). The majority of PCR-positive strains in present study belonged to serogroups previously found among isolates of cattle cases (i.e. gastrointestinal infections and diarrhea), and even found in patients with severe diseases, such as sudden infant death syndrome (SIDS), HUS, hemorrhagic colitis (HC), and urosepsis (26, 30, 31, 32). The result indicated that healthy cattle are the reservoir of \textit{E. coli} strains associated with infections and diseases.

**CONCLUSION**

Our data confirm dairy cow as important asymptomatic carrier of \textit{E. coli} strains pathogenic for humans, and as a major reservoir of atypical EPEC. To our knowledge, this is the first survey on the occurrence of these pathogenic strains in healthy cattle in China. Therefore, more epidemiological characteristics and virulence properties of these strains are necessary to be clarified in the future work.

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Huasai, S. et al.  Virulence genes of \textit{E. coli}
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