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Detections of Genes Associated with LEE and HPI Pathogenicity Island Which are Comes from Pathogenic *E. coli* of Minks

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Asbtract: To explore the pathogenesis of causeing diarrhea in mink by *E. coli*, researchers used PCR to detect the pathogenicity island genes LEE (*Ler*, *eaeA*) and HPI (*irp*, *fyuA*) of three *E. coli* whose isolation and identification of serotypes are O38, O78 and O29. Researchers designed four pairs of specific primers according to its gene sequence. The genomic DNA as the template for amplification. Results of the three *E. coli* are that *irp2* gene and *fyuA* gene of HPI were success fully amplified but the LEE (*Ler*, *eaeA*) genes were not amplified. Sequence of HPI (*irp2*, *fyuA*) showed that the homology about irp2 of the three *E. coli* is between 98.5 and 99.3%. The homology with reference *E. coli*'s *irp2* gene is between 98.5 and 99.3%.The homology about fyuA of the three *E. coli* is between 96.7 and 97.4%. The homology with reference *E. coli*'s *fyuA* gene is between 95.8 and 99.4%.

Key words: *Escherichia coli*, virulence gene, *LEE* and *HPI* gene, PCR, gene sequence

INTRODUCTION

In recent years, studies have reported that genes related with LEE and HPI pathogenicity islands are connected with the pathogenicity of *E. coli* which can cause diarrhea of animal (DaRong *et al*., 2009; Yu *et al*., 2008; WenJie *et al*., 2006). Pathogenicity island is a special area of bacterial chromosome genes encoding virulence-related genes and the virulence genes that geting from some bacteria in the evolutionary process to adapt to changes in the environment. Attaching and Effacing *E. coli* (AEEC) is similar to the Pathogenic *E. coli* (EPEC) of human intestinal, close to intestinal epithelial cell surface by the bacterial outer membrane protein factors (intimin) and other focal adhesion causing Attaching and Effacing (A/E). AEEC located in the same virulence factors concentrated area knowing as Locus of Enterocyte Effacement pathogenicity island (LEE), *E. coli* which can not cause A/E would not have LEE pathogenicity island. Ler is LEE's central regulatory genes, regulation the expression of LEE1, LEE2, LEE3, tir/eae and LEE4 (Fairbrother, 1999; Mc Danie and Kaper, 1997). AEEC can not only cause diarrhea of pig and can lead to diarrhea or death of human, rabbit, cattle and dogs. Pathogenicity island of *Yersinia* sp. named as the High Pathogenicity Island (HPI) is closely related to lethal phenotype in mice (Elliott *et al*., 1998) and in the *Yersinia* genus, it exists only in *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* and the Yersinia HPI is a large chromosomal segments to determine the level of virulence or pathogenicity has the gene of coding and synthesis which can uptaking Phytosiderophore and Yersini abactin (Ybt) and its regulatory genes, mainly responsible for iron's uptake and regulation and the genetic unit essential for expressing murine lethal phenotype.

Major structural genes *irp2* is connected with iron uptake capability of *Yersinia* and *fyuA* genes is connected with sensitivity of *Yersinia pestis*. To understand the mechanism of *E. coli* induced diarrhea in mink researchers detected LEE (Ler, eaeA) and HPI (irp2, fyuA) of the 3 isolated *E. coli* of diarrhea mink, the results are as follows:

MATERIALS AND METHODS

The tested strains: In 2011, in a mink farm of Changli, Qinhuangdao city, there is a case that Clinical diagnosis is diarrhea and deaths of weaning mink, sterile collect the dead mink's spleen and kidney as a material, crossed in the MacConkey agar plate, 37°C for 18 h, selected a single colony and then pure culture. Researchers identified it as *E. coli* by morphological characteristics, cultural characteristics and biochemical tests and numbered them as D1, D2 and D3.

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Sequence alignment of the reference strains: Included number of *E. coli* APECO1, *E. coli* O7, *E. coli*O26:H11, *E. coli*O42 and*E. coli*O83:H1 are CP000468.1, CP003034.1, AP010953.1, FN554766.1 and CP001855.1.

Main reagents: Resin type TM genomic DNA extraction kit (Beijing SBS Genetech Co., Ltd.), Dream TapTM Green PCR Master Mix 2×(Fermentas Inc.), DNA Maker (Dongsheng Bio Co., Ltd.); Primers of pathogenicity island (Sangon Biotech (Shanghai) Co., Ltd.), factor of *E. coli* O serum (China Institute of Veterinary Drugs Control, valid until October 2013) other reagents was prepared according to conventional methods.

The main instrument: PCR instrument (Dongsheng Innovation Biotech Co., Ltd.), TGL-16G high-speed centrifuge (Shanghai Anting Scientific Instrument Factory), DYY-6C electrophoresis instrument (Beijing 61 Instrument Factory), ZHWY-103B thermostatical oscillator (Shanghai Analytical Instruments Co., Ltd. Chi City), BIO-BEST200E Gel Imaging Analysis System (US West International League).

Bacterial culture: Picked colonies of *E. coli* on the slant medium then put it into 1.5 mL LB on the centrifuge tube containing nutrient broth, 37°C, 120 rpm shaking 12 h.

Extraction of genomic DNA: Take 1 mL LB broth in 1.5 mL centrifuge tube, centrifuge at the spead of 13000 rpm in 30 sec, discard the supernatant, to collect sediment; use 0.5 mL TE buffer or PBS buffer to suspend the sediment and then add 1 mL purified resin to it. Upside down 5-6 times, incubate at room temperature for 3 min, during which reversed once, 5000 rpm centrifugal 3 sec, collect the sediment; take 1 mL GN suspend the sediment, reverse mixing, 5000 rpm centrifugal 3 sec, collect the sediment; use 0.5 mL bleaching lotion rinse the purified resin twice, reversed mixing, 5000 rpm centrifuge 3 sec, collect the sediment, repeat twice; 0.8 mL anhydrous ethanol suspende the precipitation, reverse mixing, moved it to the centrifugation column, 13000 rpm centrifuge 1 min, discard the waste column into ethanol and then 13000 rpm centrifuge 1 min, try to remove ethanol; put the purification column into a clean set of 1.5 or 2 mL centrifuge tube, open it and place 2~3 min, to make ethanol to fully evaporate, add 100 μL TE buffer into the purified resin (do not stick in the wall), incubated at room temperature 3 min, 13000 rpm centrifuge 2 min. To collect a large number of genomic DNA can repeat this step 1-2 times. The collection in the centrifuge tube is the genomic DNA; take 3 μL genomic DNA to electrophoreses (1% agarose, 384V/30 min). Stored at -20°C.

Primer design

ler: Forward primer: 5'-CACACAACAAGCCCATAC, reverse primer: 5'-GATGAGTTCCG GCAGGCAA-3', the size of amplified fragment is 195 bp.

eaeA: Forward primer: 5'-TAACGGCTATTTCCGCATGA, reverse primer: 5'-TCCCAGACGATA CGATCCAG, the size of amplified fragment is 552 bp.

irp2: Forward primer: 5'-AAGGATTCGCTGTTACCGGA-3', reverse primer: 5'-TCGGCCA GGATGATT CGTCG-3', the size of amplified fragment is 301 bp.

fyuA: Forward primer: 5'-ACACGGCTT TAT CCT CTGGC, reverse primer: 5'-GGCATATTGACG ATTAACGAA-3', the size of amplified fragment is 953 bp.

PCR and sequencing: To the manual in PCR kit of Fermentas, genomic DNA as the template for PCR amplification, 25 μL PCR reaction system, there into, 12.5 μL dream TapTM green PCR master Mix, forward/reverse primer's volume(10 μmol L^{-1}) are all 0.5 μL, template's (genomic DNA) is 2 μL, Nuclease-Free Water is 9.5 μL. The LEE pathogenicity island PCR program as follows: 94°C for 5 min; 94°C for 30 sec, 58°C for 50 sec, 72°C for 90 sec, 30 amplification cycles; 72°C for 5 min. HPI Pathogenicity Island's PCR program: 94°C for 5 min; 94°C for 30 sec, 58°C for 50 sec, 72°C for 90 sec, 30 amplification cycles; 72°C for 5 min. Then, take 4 μL PCR products into 1% agarose for electrophoresis, PCR products were sent to Sangon Biotech (Shanghai) Co., Ltd. for gene sequencing.

Identification of *E. coli* serotype: Inoculate three strains of bacteria in the ordinary agar slant tubes, set at 37°C for 24 h, use 2 mL 0.5% phenol saline to wash the ordinary agar slant culture, made up of thick bacterial suspension, high-pressure sterilization for 2 h, destroy their "k" antigen, made it into high-pressure antigen. Then, use a variety of standard single factors to proceed the glass plate agglutination meanwhile, a mixture of antigen and 0.5% phenol saline as control, to observe whether have the phenomenon of self-curing. Occuring clear agglutination (++) within half a minute was judged as positive.

RESULTS

Extraction of genomic DNA: Useing the resinous type™ genomic DNA kit extracte the genomic DNA of *E. coli*, obtained genomic DNA bands, shown in Fig. 1 indicating a successful extraction of colibacillary genomic DNA.

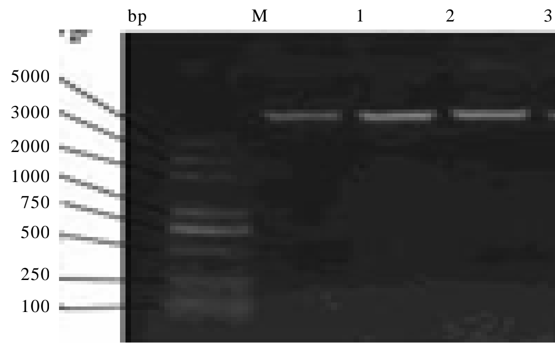

Fig. 1: Salmonella genomic DNA; M: Maker DL5000; 1-3: D1, D2, D3 DNA

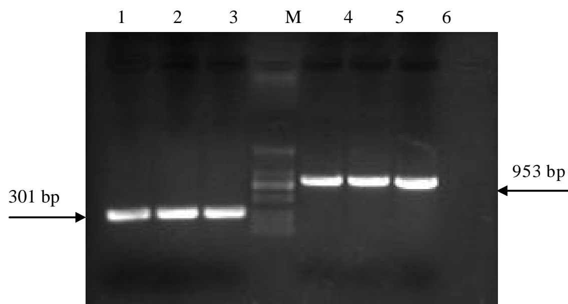

Fig. 2: PCR amplification result of *irp2* and *fyuA* gene from *E. coli.* M: Maker DL2000; 1-3: D1, D2, D3 *irp2* gene; 4-6: D1, D2, D3 *fyuA* gene

PCR amplification of virulence genes: Utilize *E. coli* genomic DNA as template researchers amplifed about the size of 301 and 953 bp specific band shown in Fig. 2, the results are basically consistent with the expected size indicating that successfully amplified and detected *E. coli irp2* and *fyuA* gene but did not amplified LEE (Ler, eaeA)'s specific bands.

Gene sequencing of PCR products: Sent the PCR products of *irp2* and *fyuA* gene to Shanghai Chemical Biotechnology Co., Ltd. The sequencing results determined the *irp2* gene sequences of three *E. coli* and *fyuA* gene sequences of other three *E. coli.*

Identification of *E. coli* serotype: Utilize *E. coli*'s single factors to proceed the glass plate agglutination, determined the serotype of D1 is O78, the serotype of D2 is O29, the serotype of D3 is O38.

Homology of pathogenicity island in different serotype *E. coli*: Comparison of *irp2* gene sequences between the measured and NCBI GenBank sequences have been published showed that: Homology of three test strains (D1, D2 and D3) of the *irp2* gene sequences and reference

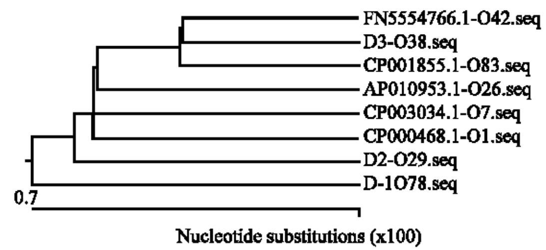

Fig. 3: The phylogenetic tree of nucleotide sequence of *irp2* genes

Table 1: Sequences homologous analysis of *irp2* gene from *E. coli*

Percent identity	1	2	3	4	5	6	7	8
1	-	99.6	100.0	100.0	100.0	99.2	98.9	99.2
2	0.4	-	99.6	99.6	99.6	98.9	98.5	98.9
3	0.0	0.4	-	100.0	100.0	99.2	98.9	99.2
4	0.0	0.4	0.0	-	100.0	99.2	98.9	99.2
5	0.0	0.4	0.0	0.0	-	99.2	98.9	99.2
6	0.8	1.1	0.8	0.8	0.8	-	99.3	99.3
7	1.1	1.5	1.1	1.1	1.1	0.7	-	98.5
8	0.8	1.1	0.8	0.8	0.8	0.7	1.5	-

1: FN554766.1-O42.seq; 2: AP010953.1-O26.seq; 3: CP000468.1-O1.seq; 4: CP001855.1-O83.seq; 5: CP003034.1-O7.seq; 6: D3-O38.seq; 7: D1-O78.seq; D2-O29.seq

Table 2: Sequences homologous analysis of *fyuA* gene from *E. coli*

Percent identity	1	2	3	4	5	6	7	8
1	-	99.3	99.4	99.0	99.1	99.3	99.4	96.7
2	0.2	-	98.6	98.1	98.2	98.5	98.6	97.4
3	0.0	0.2	-	99.6	99.7	99.9	100.0	95.9
4	0.4	0.7	0.4	-	99.5	99.5	99.6	95.5
5	0.3	0.6	0.3	0.6	-	99.6	99.7	95.7
6	0.1	0.3	0.1	0.6	0.4	-	99.9	95.8
7	0.0	0.2	0.0	0.4	0.3	0.1	-	95.9
8	1.0	0.8	1.0	1.5	1.1	1.1	1.0	-

1: D3-O38.seq; 2: D1-O78.seq; 3: FN554766.1-O42.seq; 4: AP010953.1-O26.seq; 5: CP000468.1-O1.seq; 6: CP001855.1-O83.seq; 7: CP003034.1-07.seq; 8: D2-O29.seq

strains (O1, O7, O26, O42 and O83) of the *irp2* gene sequence is 98.5~99.3% and the homology of three test strains D1, D2 and D3 is 98.5~99.3% shown in Table 1. According to the principles of homology groups, researchers constituded the phylogenetic trees of *E. coli.* From the evolutionary tree of *irp2* gene researchers can see that *irp2* gene listed in the different serotypes of *E. coli* is divided into four groups, O78 of D1 strain as group I, O29 of D2 strain as II group, O38 of D3 strains and O83 of O42 for the group III. Genetic relationship between D2 and D3 strains is close and between D2 and D1 strains is distantly related shown in Fig. 3.

FyuA sequence between three test strains D1, D2 and D3 and strains O1, O7, O26, O42 and *E. coli* O83 from GenbBank is 95.8~99.4%, homology of the test strains is 96.7~97.4% shown in Table 2; seeing from the evolutionary tree researchers can konw that listed in the different serotypes of *fyuA* gene is divided into four

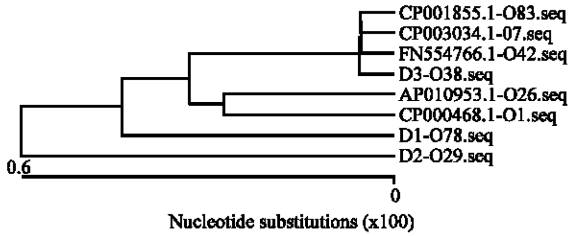

Fig. 4: The phylogenetic tree of nucleotide sequence of *fyuA* genes

groups, O78 of D1 strain as group I, O29 of D2 strains as the II group and O38, O7, O42 and O83 of D3 strain as III group. Genetic relationship between D1 and D3 strains is close between D1 and D2 strains is distantly related shown in Fig. 4.

According to the results of homologous fragment analysis between *irp2* and *fyuA* 2 genes of *E. coli*, researchers can draw a conclusion that three strains' HPI pathogenicity island related genes like *irp2* and *fyuA* island gene fragments and in GenBank reference strains of *irp2* and *fyuA* gene nucleotide sequence is highly homologous indicating that HPI pathogenicity island *irp2* and *fyuA* genes less mutate under normal circumstances. So, the virulence of *E. coli* having HPI pathogenicity island *irp2* and *fyuA* genes is difficult to mutate, the result is not only shows the HPI pathogenicity island *irp2* and *fyuA* genes exist in *E. coli* of diarrheal mink but also shows that the occurrence of mink's diarrhea and death are closely related with *irp2* and *fyuA* genes.

DISCUSSION

The HPI pathogenicity island of *Yersinia enterocolitica* is located in the chromosome, its size is 45 kb. The main structure of the pathogenicity island is irp2 and fyuA. Irp2 is connected with encodeing of the polymer protein HMWP2 and iron uptake ability of Yersinia, fyuA encoding avermectin receptor of plague, relate to the sensitivity of Yersinia pestis. rp2 can as a sign of detection HPI pathogenicity island (Carniel *et al*., 1996).

HPI is not only exist in lethal *Yersinia pestis* of mice but also can be transferred to other bacteria's genne of Enterobacteriaceae and has become the important virulence factors of humanized, bovine's and rabbit's pathogenic *E. coli* (Chen *et al*., 2006; Shao-Hua and Tong-Jie, 2004) however, about pathogenicity island of young mink's pathogenic *E. coli* is still less reported. In this study, HPI was detected in three different serotypes of *E. coli* and sequence analysis also proved that the *E. coli* sequence of irp2 and fyuA of experimental weaning Aberdeen inspectors is highly homologous with the homologous sequence in GenBank. HPI pathogenicity islands are highly pathogenic virulence island, its very high rate in the detection of mink's HPI pathogenicity island of *Escherichia coli*. In the related experiments, the detection rate of chicken pathogenicity island of *Escherichia coli* has been reached 75%, researchers are still detected out HPI pathogenicity island of *E. coli* in commercially available pork and beef indicating that livestock and poultry may be the host of this type of pathogenic *E. coli* and its potential epidemiological significance can not be ignored. Worth further study.

Bacterial virulence factors has unusual close contact withcomponents of exogenous DNA, although parenthesis of some types of foreign DNA does not participate in encoding the virulence of the bacteria, it also has important medical significance (Nataro and Kaper, 1998). At both ends of the island of bacterial virulence have insertion sequence elements and repeat sequence, suggesting the possibility of the existence of recombinant DNA which may also be an important driving force of bacterial evolutionary (Hacker *et al*., 1997). Different serotypes of *E. coli* is determined by *E. coli* O-antigen (Bastin and Reeves, 1995). Classified according to O-antigen, there are 180 different *E. coli* serotypes now (Whitfield, 1995; Reeves and Wang, 2002; Stenulz *et al*., 2006). On trail, the serotypes of *Escherichia coli* which can cause diarrhea in mink are O78, O38, O26, however, researchers also isolated *E. coli* carrying *irp2* and *fyuA* genes in diarrhea of cattle, pigs, chickens, rabbits, raccoon and others, O78, O38 have higher rate of separation in 18 O serotypes, guessing that the serotypes of *E. coli* caused animals' diarrhea in Qinhuangdao are mainly O78, O38.

CONCLUSION

According to comparative results, the majority differences between nucleotide is mainly in the different between the single nucleotide (individual two nucleotides), did not appear for several nucleotide which means that no large fragments of foreign DNA insertion happend in experimental strains. High homology indicating little variation between the strains. Research from the pathogenic mechanism of pathogenic bacteria, pathogenic factors and other aspects will fundamentally promote the development of prevention and control measures for *E. coli* disease and solve clinical problems. Highly pathogenic virulence island of pathogenic *E. coli* as an emerging important virulence factor making the process and mechanism of pathopoiesia becomes more complex, its further study will be a new focus. For this

study, from roughly single channels and methods to diagnosis, prevention and treatment of scale, prevention and treatment of diarrhea in mink have some practical significance, provided clues for this prevention.

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