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# Prevalence of the "High-pathogenicity Island" in *Escherichia coli* Isolated from Clinical and Subclinical Bovine Mastitis in China

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## ABSTRACT

The aim of this study was to investigate the distribution of high pathogenicity island (HPI) in *Escherichia coli* (*E. coli*) isolated from cows with clinical and subclinical mastitis in China. Milk samples from 750 cows with clinical and subclinical mastitis in seven provinces were used to screen Gram-negative bacteria by MacConkey agar. 115 *E. coli* isolates were further identified from the Gram-negative bacteria by microbiological tests. Total DNA extraction from *E. coli* was used to detect HPI genes by PCR. The PCR results showed that in 115 samples, 29, 22 and 20 were positive for genes *irp2*, *fyuA* and *intB*, respectively. Sequence analysis of randomly selected 10 PCR products showed that homology of genes *irp2*, *fyuA* and *intB* were 97.1, 98.2 and 97.2% identical to the published sequences. In addition, $HPI^+$ isolates were associated with *fyuA* (75.86%) and *asn*_tRNA locus (68.96%). We further analyzed the association of the prevalence of HPI and O serotypes and found that HPI occurred in several serotypes including $O_{149}$, $O_{93}$, $O_9$ and $O_7$. Therefore, we concluded that HPI was widely distributed in the *E. coli* isolates from cows with clinical and subclinical mastitis in China. The prevalence of HPI is associated with special serotypes in *E.coli* from cows with clinical and subclinical mastitis.

**Key words:** Clinical mastitis, HPI genes, microbiological tests, O serogroups, cows

## INTRODUCTION

High-pathogenicity island (HPI), a large chromosomal DNA fragment of *Yersinia pestis,* is highly associated with the mouse-lethal phenotype of *Yersinia* (Bearden *et al*., 1997; Buchrieser *et al*., 1998a; Alvarez and Cardineau, 2010; Carniel *et al*., 1996). HPI carries the gene *fyuA*, which is specific for the pesticin receptor and the iron repressible protein (*irp*) loci encoding the siderophore yersiniabactin (Ybt). The functional core of HPI is called *irp2-fyuA* gene cluster, the major gene element encoding an iron uptake system mediated by Ybt. The Ybt is associated with asparagine-specific tRNA loci and carries an integrase gene, *int*, often associated with a phage genome (Carniel *et al*., 1996; Rakin *et al*., 1999; Gehring *et al*., 1998; Buchrieser *et al*., 1998b).

*Irp2*, one of the major structural genes of the core conservative region of HPI, has been used as a marker to detect HPI. *FyuA* gene coded for the yersiniabactin receptor FyuA and also served as a receptor for pesticin (Lucier *et al*., 1996; Rakin *et al*., 1994). Besides pathogenic *Yersinia* species, HPI has been reported as a part of the genomes of other enterobacteria such as *Klebsiella* spp., *Citrobacter* spp. and *Escherichia coli* (*E. coli*) (Hacker *et al*., 1999; Schubert *et al*., 1998).

Dairy cattle with clinical mastitis caused by *Escherichia coli* exhibits a wide range of disease severity, from mild, with only local inflammatory changes of the mammary gland, to severe, with significant systemic derangement. *Escherichia coli* is one of the commonest causes of bovine clinical mastitis (Green *et al*., 2005) and a major problem in lactating dairy cows (Kobori *et al*., 2004). It has been reported that the incidence of *E. coli* mastitis has recently increased in some countries (Green *et al*., 2005; Guler and Gunduz, 2007). Numerous studies in identifying virulence factors of *E. coli* isolated from cows with clinical mastitis have been conducted (Barrow and Hill, 1989; Kaipainen *et al*., 2002). However, the role of HPI in the *E. coli* caused bovine mastitis is still unclear. The present study has detected the prevalence of HPI using specific HPI markers from 115 *E. coli* isolated from milk samples, which were collected from 750 cows with clinical and subclinical mastitis in different areas of China.

## MATERIALS AND METHODS

**Bacterial isolation:** Milk samples from 750 cows with clinical and subclinical mastitis in different dairy farms of seven provinces (Beijing, Inner Mongolia, Gansu, Sichuan, Chongqing, Guizhou and Yunnan) in China were collected between 2008 and 2009. Clinical and subclinical mastitis were identified by the Lanzhou Mastitis Test (LMT) and clinical examination (Li *et al*., 2002). Approximately 5 mL of milk were collected in sterile vials after disinfection with 70% ethanol and removal of the first 3 to 4 streams of milk. Samples were cultured in MacConkeys (MAC) agar. The isolates were identified as *E. coli* based on colony morphology and color, Gram stain and API-20E Enteric Identification System (Wenz *et al*., 2006) and stored at -70°C.

**Identification of O-serotyping:** Pure cultures of bacterial strains (*Escherichia coli* isolates) were grown on nutrient agar slants and sent to Bacterial products testing center, China Institute of Veterinary Drugs Control, (Beijing, China) for serotyping.

**Bacterial pre-culturing and extraction of DNA templates:** Each bacterial strain (*Escherichia coli* isolates) grown in LB agar at 37°C for 24 h was selected single colony and transfered into LB nutrient broth overnight with vigorous agitation at 37°C. The bacterial culture was enriched by centrifugation at 12000 g and DNA samples were extracted using a bacterial genomic DNA extraction kit (from *TaKaRa* Biotechnology Co., Ltd. (Dalian, P.R.China)). The productions were used directly or stored at -70°C before PCR as DNA templates.

**PCR detection of HPI in *E. coli*:** The specific PCR primers were respectively designed according to the published gene sequences of *irp2*, *fyuA* and *asn_tRNA_intB* in Genebank. PCR assays were performed by the Applied Biosystems (2720 Thermal Cycler America). The primers were synthesized by Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, P.R. China). All the PCR reagents were purchased from *TaKaRa* Biotechnology Co., Ltd. (Dalian, P.R. China). *Irp2*, *fyuA* and *asn_tRNA_intB* genes were detected using the three pairs of specific primers as following protocols.

The PCR assay was carried out in a total volume of 50 μL of mixture containing 5 μL of 10x PCR buffer ($Mg^{2+}$), 5 IU of *Taq* polymerase, 4 μL of dNTP mixture (each 2.5 mmol $L^{-1}$), 1 μL of HPI primers set (each 50 mmol $L^{-1}$), 2 μL of DNA template and deionized water to a final volume of 50 μL. The amplified DNA products were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide and detected under ultraviolet light. The expected sizes of PCR products should be 276, 1071 and 1512 bp, respectively. The sequences of the forward (FP) and reverse (RP) primers used for PCR reactions and PCR amplification Tm were listed in Table 1.

**DNA sequencing and sequence comparison:** Ten PCR products from *irp2*-positive, *fyuA*-positive or *intB*-positive *E. coli* strains were randomly selected for amplification. The amplified DNA products were purified and connected to the pMD18-T carrying agent, then which were transformed into *E. coli* JM109 and cultured. Eventually, positive clones were selected to extract plasmid DNAs and identified by PCR. DNA sequencing was performed by Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, P.R.China). The nucleotide sequences were analyzed with the DNAStar and BLAST in NCBI.

## RESULTS

**Survey for the presence of *irp2, fyuA* and *intB* genes among *E. coli* strains:** We isolated 115 *E. coli* strains from a total of 750 mastitc milk samples. After analyzing all three HPI-genes *irp2, fyuA* and *intB* in each of 115 *E. coli*, found that 22 (19.13%) of the 115 *E.coli* strains harbored both *irp2* and *fyuA* gene at the same time, while 7 (6.09%) of that only harbored genes *irp2*. Therefore, totally 29 (25.22%) *E. coli* isolates were HPI-positive strains. In addition, in the 29 HPI-positive *E. coli*, 20 (68.96%) strains were *intB* postitive, indicating that most HPI-positive *E. coli* isolates carried the *intB* gene and the HPI was linked at *asn*_tRNA locus (Fig. 1-3).

**Sequence analysis of the genes of *irp2, fyuA, asn_tRNA_intB* in *E. coli*:** After analyzed by DNAStar and MEGA programs, we found that all 10 *irp2* gene sequences were 276 bp and the sequence homology was identical to the published *irp2* sequences (97.1%). The sequence homology of 5 $irp2^{+}fyuA^{+}intB^{+}$ strains and 3 $irp2^{+}fyuA^{-}intB^{+}$ strains and 2 $irp2^{+}fyuA^{-}intB^{-}$ strains exceeded 98.3%. The *fyuA* gene sequences were 1071 bp and sequence homology was identical to the published *fyuA* sequences (98.2%). The *asn*_tRNA_*intB* gene sequences were 1512 bp and sequence homology was identical to the published *asn*_tRNA_*intB* sequences (97.2%). The phylogenetic tree of isolated strains on the genes of *asn_tRNA_intB* in *E. coli* was seen (Fig. 4-6).

**HPI and O-serotyping in *E. coli*:** For the 115 *E. coli* strains, 63 isolates were identified as 39 serogroups, 3 isolates were self-clumpinged and 49 isolates could not be serotyped. As we shown (Table 2), $O_{93}$, $O_{9}$, $O_{146}$, $O_{7}$, $O_{74}$ serotypes were the most prevalent serogroups. Among the 39

Table 1: Oligonucleotide primers used in the study

| Primer | Primer sequence (5'-3') | Target gene | Annealing temperature (°C) | PCR fragment size (bp) |
|---|---|---|---|---|
| Irp2-L | AAGGATTCGCTGTTACCGGAC | *Irp2* | 55 | 276 |
| Irp2-R | TCGTCGGGCAGCGTTTCTTCT | | | |
| FyuA-L | CCGTCTTACAGGGACTCACAACAAT | *fyuA* | 58 | 1071 |
| FyuA-R | GGTACAGCCCAAACACCATATCAAC | | | |
| intB-L | GTGTGAAAACTCTTCTCGGTGC | *asn*_tRNA_*intB* | 60 | 1512 |
| intB-R | GTCGCTCTTTCATTCCTCTGTG | | | |

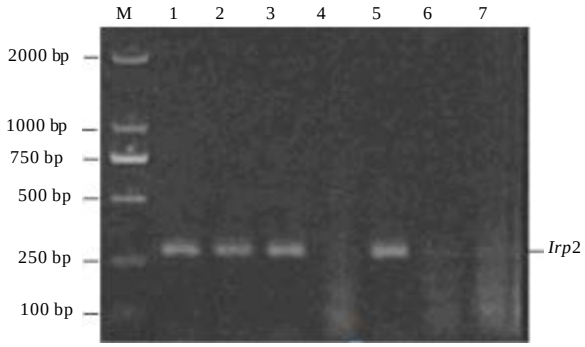


Fig. 1: PCR for detection *irp2* in partial isolates, M. eDNA Marker DL2000, 1. S433014 strains; 2, 3 and 5; *irp2*$^{+}$ strains; 4, 6 and 7; *irp2*$^{-}$ strains

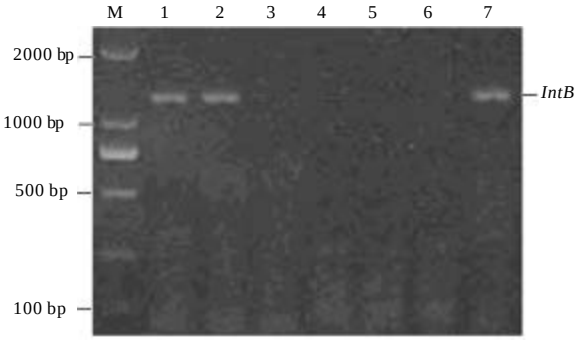


Fig. 2: PCR for detection *fyuA* in partial isolates, M. DNA Marker DL2000, 1. S433014 strains, 2, 3, 4, 5 and 6; *fyuA*$^{+}$ strains, 7. *fyuA*$^{-}$ strains

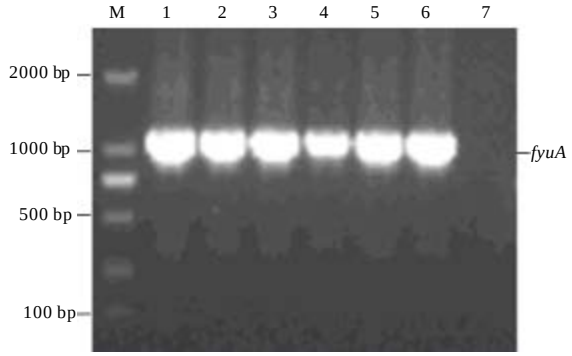


Fig. 3: PCR for detection *asn_tRNA_intB* in partial isolates, M. DNA Marker DL2000, 1. S452621 strains, 2 and 7: *asn_tRNA_intB*$^{+}$ strains; 3, 4, 5 and 6: *asn_tRNA_intB*$^{-}$ strains

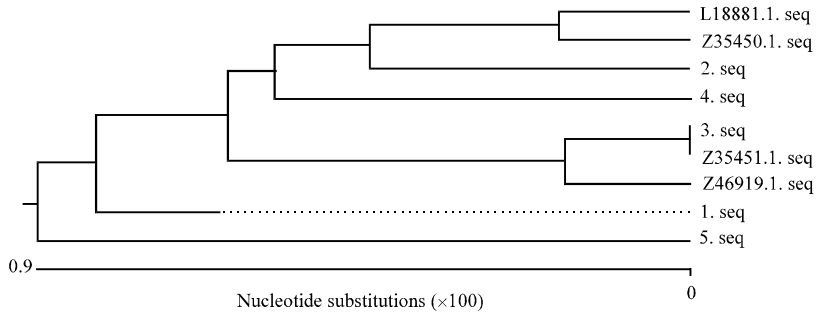


Fig. 4: The phylogenetic tree of isolated strains on *irp2* gene sequences, 1, 2, 3, 4, 5. seq. isolated strains *irp2* gene sequences

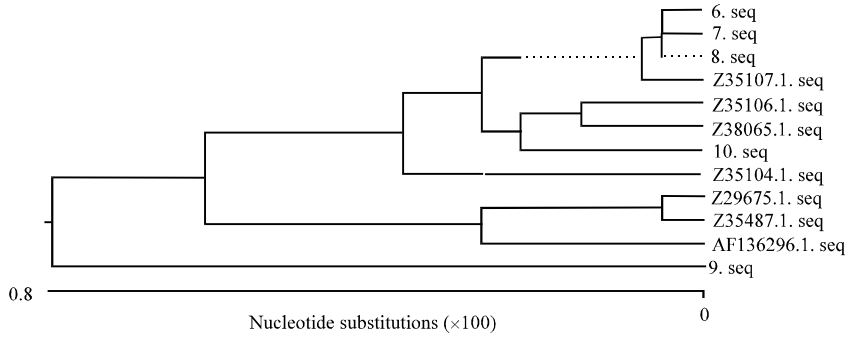


Fig. 5: The phylogenetic tree of isolated strains on *fyuA* gene sequences, 6, 7, 8, 9, 10. seq. isolated strains *fyuA* gene sequences

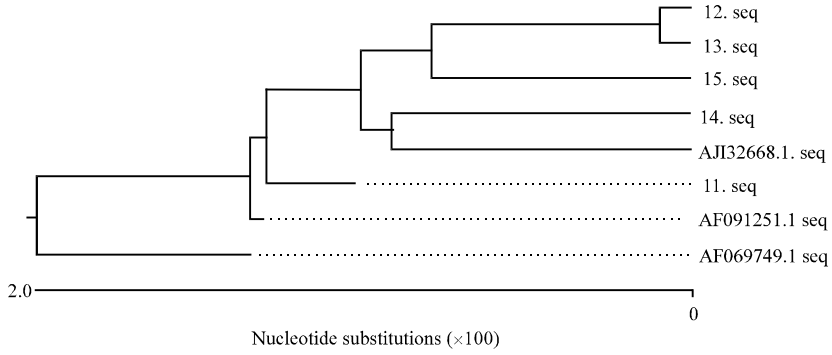


Fig. 6: The phylogenetic tree of isolated strains on *asn_tRNA_intB* gene sequences, 11, 12, 13, 14, 15. seq. isolated strains *asn_tRNA_intB* gene sequences

serogroups, 22 HPI-positive strains belongs to 16 different serogroups, 6 HPI-positive strains were non-serotyping and 1 was self-clumpinged. Furthermore, comparing to the detection rates of HPI in the most prevalent serogroups, $O_{93}$ serogroup was the highest one than $O_9$ (33.3%), $O_7$ (33.3%), $O_{74}$ (33.3%) and $O_{146}$ (20%). While among non-prevalent serogroups, $O_{149}$ serogroups were 100% HPI-positive strains.

Table 2: Relation statistic between HPI$^+$ and isolates serotype

| Serotype | Positive ratio* | Serotype | Positive ratio |
|---|---|---|---|
| $O_{93}$ | 4/9 | $O_{36}$ | 0/2 |
| $O_9$ | 2/6 | $O_{61}$ | 0/2 |
| $O_{146}$ | 2/5 | $O_{66}$ | 1/2 |
| $O_7$ | 1/4 | $O_{74}$ | 1/2 |
| $O_{10}$ | 0/3 | $O_{83}$ | 0/2 |
| $O_{21}$ | 0/3 | $O_{107}$ | 1/2 |
| $O_{22}$ | 0/3 | $O_{149}$ | 2/2 |
| $O_{32}$ | 0/2 | Others | 15/66 |

*Ratio = No. of HPI positive isolates/No. of isolates

## DICUSSION

HPI was first discovered in pathogenic *Yersinia* strains and has recently been found in other enterobacteria. Schubert *et al.* (1998) demonstrated that the *fyuA-irp* gene cluster is absent in *Shigella*, *S.enterica* serotypes Enteritidis and Typhimurium and Shiga toxin-producing *E.coli* (EHEC). The gene cluster is infrequently detected in *E.coli* strains of EPEC, ETEC and EIEC pathotypes. Strikingly, the EAEC pathotype, which causes acute and chronic diarrhea in infants (Huppertz *et al.*, 1997; Frederick, 2011; Naidu *et al.*, 2007), frequently harbors a chromosomal *fyuA-irp* gene cluster (Buchrieser *et al.*, 1998a; Hacker *et al.*, 1999; Schubert *et al.*, 1998). The HPI of *Yersinia* spp. is responsible for lethality for mice and for biosynthesis and uptake of the siderophore yersiniabactin. Yersiniabactin-mediated iron acquisition is thought to be the main function of the HPI (Gehring *et al.*, 1998) and genes involved in yersiniabactin biosynthesis, transport and regulation (*irp1* to *irp9*, *ybtA* and *fyuA*) are clustered on the "core" of the HPI. Although, HPI was the important virulence factor of pathogenicity *E. coli* isolates from humans, piglets and rabbits (Clermont *et al.*, 2001; Bach *et al.*, 2000; Penteado *et al.*, 2002), there is no study reporting HPI of *E. coli* isolates from bovine mastitis milk in China. This is critical problem for dairy industry in China now.

The *irp2* and *fyuA* genes are the two major structure of the "core" of the *Yersinia* HPI. Both of them are extremely conservative and usually exist at the same time. In our study, we found that 22 (19.13%) of the strains harbored both *irp2* and *fyuA* gene at the same time, while 7 (6.09%) of that only harbored the *irp2* genes. Totally 29 (25.22%) *E. coli* isolates were HPI-positive strains. The detection rate is lower than that from humans (32.25-34.9%) (Yong *et al.*, 2002; Changyun and Jianguo, 2000) and avians (44.9%) (Wenjie *et al.*, 2006), but higher than that from pigs (12.7-16.66%) (Xiang *et al.*, 2006; Darong *et al.*, 2006). These results indicate that horizontal gene transfer of HPI occurs between *Yersinia* strains and *E. coli* strains and *Yersinia* HPI was widespread in *E. coli* isolates from bovine mastitis milk and confered virulence-associated functions. The data of results also offered certain evidences for horizontal gene transfer of *Yersinia* HPI. The results of our survey indicated that the *irp2* and *fyuA* genes may not be simultaneously expressed in one strain. Because of the *fyuA* gene was only detected in 75.86% of HPI-positive strains. The absence of *fyuA* gene, may reduce the iron-uptaken ability and subsequently attenuate the virulence for these HPI-positive strains without *fyuA* gene. This reduced virulence may result from gene mutation or gene recombination in the course of horizontal gene transfer of HPI among bacterial species for adaptation to a new environment. The functions of the *fyuA-irp* gene cluster in *E. coli* strains still remains unclear.

The HPI in *Yersinia* is located next to the *asn* tRNA bacterial attachment site. The *asnT* locus is linked to a gene which is highly homologous with a phage derived integrase determinant termed *int* that codes for a P4-like integrase. The instability of *Yersinia* HPI might be due to containing some mobile elements in *Yersinia* HPI, such as potential insertion sequences and integrase. Partial deletion of *int* might result in a non-functional integrase and incline to stabilization of the HPI in the chromosome of those isolates (Schubert *et al.*, 1999). In our research, 64% of *Yersinia* HPI-positive strains from bovine mastitis milk carried the *intB* gene and inserted the *asn*-tRNA site. This is in line with some reports that *Yersinia* HPI from humans and pigs mostly linked to the *asn*-tRNA site (Bach *et al.*, 2000; Wenjie *et al.*, 2006). As recent report from Germany demonstrated that the HPI of $O_{26}$ STEC strains and two *E. coli* strains isolated from blood poisoning avians had same deletion at its left junction, leading to a truncated integrase gene *int* (Karch *et al.*, 1999). Therefore, we conclude that the HPI of the STEC $O_{26}$ group represents a new and unique type of HPI with a partially deleted *int*. The deletion in the *int* gene may result in a nonfunctional integrase and subsequent fixation of the HPI in the genome of this STEC clonal lineage.

We also demonstrated that $O_{93}$, $O_9$, $O_{146}$, $O_7$ serotypes were dominant serotypes in all *E. coli* isolated from seven provinces in China. However, the *Yersinia* HPI was expressed differently among these different dominant serotypes and all two isolates of non-dominant serotype $O_{149}$ are HPI positive. These results indicate that the distribution of *Yersinia* HPI existed distinction among different serotype strains. HPI might be only associated with special serotypes, not with dominant serotypes.

In summary, the findings in this study demonstrate that *Yersinia* HPI was widely distributed in the *Escherichia coli* isolates from cows with clinical and subclinical mastitis in China. The prevalence of HPI is probably associated with special serotypes in *E. coli* from cows with clinical and subclinical mastitis.

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