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Phylogenetic Analysis and Detection of Related Genes of High-Pathogenicity Island of *Escherichia coli* Strains Isolated from Chicken in Hebei Province, China

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Abstract: The detections of the Locus of Enterocyte Effacement Pathogenicity Island (LEE) and High Pathogenicity island (HPI) in 26 *E. coli* samples from chickens were conducted by PCR. *Ler* and *eaeA* genes located in the core of LEE Virulence Island while *irp2*, *fyuA* and *asn_RNA_intB* genes in HPI Pathogenicity Island were all detected. At the same time, O serotype identification of 26 *E. coli* samples were conducted. The results showed that the positive rate of *Ler* gene in LEE Virulence Island was 3.85% (1/26) while eaeA was 3.85% (1/26). The rate of *fyuA* gene in HPI pathogenicity island was 42.3% (11/26) while *irp2* was 73% (19/26), *irp2+fyuA+* was 42.3% there was no *asn_RNA_intB* gene. The results of serotype identifications showed that 15 strains related to serotype O4, O91, O78, O107, O38, O111, O88, O53, O24, O9 and O11 except 11 strains. Among 15 strains, O91, O78 and O38 were dominant serotypes whose proportion were 20 (3/15), 20 (3/15) and 26.7% (4/15), respectively. The nucleotide sequence similarities were >95% for 26 strains isolated from chickens.

Key words: *E. coli*, Virulence Island, LEE, HPI, serotype

INTRODUCTION

Virulence Island (or Pathogenicity Island) is a chromosomes DNA fragment having relative major molecular weight which encoding bacteria virulence gene cluster (Koczura *et al*., 2012). It produces a marked effect during the process of new pathogenicity bacteria occur. LEE Virulence Island (Locus of Enterocyte Effacement Pathogenicity Island) is a 35.5 kb gene groups located on the chromosomes, encoding virulence factor, containing all of the genes encoding A/E damnification and other virulence genes (Nei, 1978). EaeA is the most important gene causing the damage of A/E. The bacteria outer membrane protein (or intimin) encoded by *EaeA* gene mediates the compact adhesion between bacteria and enterocyte. Ler is the center regulation gene and the activating transcription factor encoded by LEE Virulence Island. HPI pathogenicity island was firstly discovered in the *Yersinia* genus. It is named high Pathogenicity Island (Jin *et al*., 2005; Carniel *et al*., 1996; Mokracka *et al*., 2004) because it is closely related to the mouse lethal phenotypes of *Yersinia* genus.

It contains the genes and regulatory genes of coding the synthesis and uptake of ironophore and Yersini abactin (Ybt). HPI has the major function of regulation and iron uptake, *irp2* gene can be the detection sign of HPI (Cheng *et al*., 2000; Cui *et al*., 2001). *Irp2* gene is related to the uptake ability of ironophore while fyuA is related to the sensitivity of Yersinia pesticine. HPI can insert into the asn-tRNA site of *E. coli* genome by the integrase encoded by int so, researchers can determine whether it is intergrased into the site through the detection of HPI. Virulence Island and toxic protein encoded by Virulence Island are the major reasons causing animals diseases.

This study amplified the relative genes in Virulence Island of 26 *E. coli* strains from chickens through designing primer and consulting relative references. Meanwhile, the differences among Virulence Island of different serotypes of *E. coli* were conducted. It is very important to research the epidemiology of pathogenicity *E. coli* and provide scientific basis for studying pathogenicity further.

MATERIALS AND METHODS

Tested strains: The 26 *E. coli* chickens samples isolated from chicken and internal organs in Hebei Province, conserved in Key Laboratory of Preventive Veterinary Medicine in Hebei and numbered 1-26. The reference

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strains for irp2 sequence were um146, 10407, 83972, NA114 and O83H1 for fyuA were um146, 10393, APEC01, S88. All of them were from Genebank.

Culture of bacteria in LB broth: All the *E. coli* strains inoculated in LB broth by sterile operation then cultured in thermostatic culture scillator to stay overnight.

Extraction of *E. coli* genome DNA: The 1.0 mL LB broth *E. coli* bacterium was placed into 1.5 mL sterile centrifuge tube, centrifuged at 13000 rpm for 30 sec to collect thalline, Thalline was added into 0.5 mL TE buffer, mixed with 1 mL purification resin, reversed for 5-6 times. Incubated at room temperature for 3 min, reversed one time during the incubation then centrifuged at 5000 rpm for 3 sec to collect sediment 1 mL GN combined liquid mixed with purification resin, reversed and centrifuged at 5000 rpm for 3 sec to collect sediment. The 0.5 mL rinse solution rinsed purification resin for two times then repeated the above operation once, 0.8 mL absolute alcohol with the sediment were placed into centrifugal purification column and then centrifuged at 13000 rpm for 1 min, discarded the waste solution, centrifuged for 1 min to remove alcohol entirely. The purification column was placed into a clean 1.5 mL centrifuge tube, 100 uL TE buffer was added into purification resin, stay for 3 min at room temperature then centrifuged at 13000 rpm for 2 min. Thus, the liquid collected in the centrifuge tube was genome DNA, conserved at -20°C. The 2 uL samples was tested by electrophoresis (1% agarose, 120 V, 20 min), 2 uL genome DNA was tested (110 V) for 30 min by electrophoresis then observed and record the results.

Design primers: The 5 pairs specific primers of LEE (ler and eaeA) and Yersinia HPI (irp2, fyuA and asn_RNA_intB) were designed according to the reported *E. coli* virulence island gene sequences from genbank, the sizes of five objective fragments were 195, 552, 301, 953 and 1391 bp, respectively. The synthesis of primers was conducted by Beijing SBS Genetech Co., Ltd. The sequences were shown under Table 1.

Table 1: Sequences of PCR primers

Primer name	Primer sequence	Objective gene	Size of objective fragment (bp)
ler-1	cgc aca caa caa gcc cat ac	*ler*	195
ler-2	gat gag ttc cgg cag gca a		
intimin-1	taa cgg cat ttt ccg cat ga	*eaeA*	552
intimin-2	tcc cag acg ata cga tcc ag		
irp2F	aag gat tcg ctg tta ccg ga	*irp2*	301
irp2R	tcg gcc agg atg att cgt cg		
fyuA-1	aca cgg ctt tat cct ctg gc	*fyuA*	953
fyuA-2	ggc ata ttg acg att aac gaa		
intB_L	gaa cgg cgg act gtt aat	*asn_RNA_intB*	1391
intB_R	atc gct ttg cgg gct tct agg		

PCR amplification was conducted by means of genome DNA template: The total amplification volume was 25 uL including PCR Master Mix 12.5 uL each primer was 0.5 uL the genome DNA template 2 μL and Nuclease-Free Water 9.5 uL. PCR conditions consist of denaturation for 5 min at 94°C followed by 30 cycles of 94°C for 30 sec, 58°C for 50 sec, 72°C for 90 sec and then extension at 72°C for 5 min.

Sequence determination of PCR amplification: Selected the representative product of PCR amplification and determined the sequences in Beijing SBS Genetech Co., Ltd.

Serotype identification of *E. coli*: Picked the tested colony and then inoculated on bevel tubule at 37°C for 24 h. The 2 mL 0.5% phenol physiological saline douched the bevel culture into the round bottom tube, autoclaving for 2 h in order to destroy K antigen. The 14 standard single factor serum were carried by glass plate agglutination reaction, the control was admixture of phenol physiological saline and high pressure antigen. If there was obvious agglutination within 0.5 min which showed positive or no agglutination showed negative.

RESULTS

Extraction of *E. coli* genome DNA: According to the instruction of extraction of resin genome DNA kit, the objective fragment were observed (Fig. 1).

PCR Amplification of virulence island

Amplification of LEE: The two electrophoresis strips (195 and 552 bp) were detected by PCR according to the two specific primers of LEE (Ler and eaeA) confirmed with the expected fragment (Fig. 2 and 3).

Amplification of Yersinia HPI: The 301 and 953 bp electrophoresis strips were detected by PCR according to the two specific primers of HPI (irp2 and fyuA) there was no *asn_RNA_intB* gene. Which was confirmed with the expected fragment (Fig. 4 and 5).

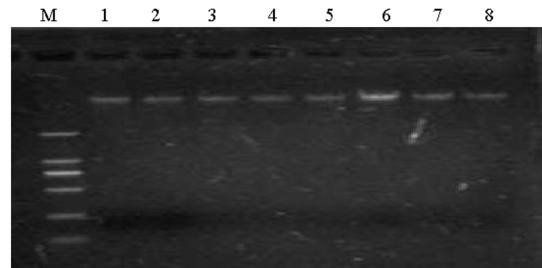

Fig. 1: *E. coli* genomic DNA results. M: DM2000 Marker

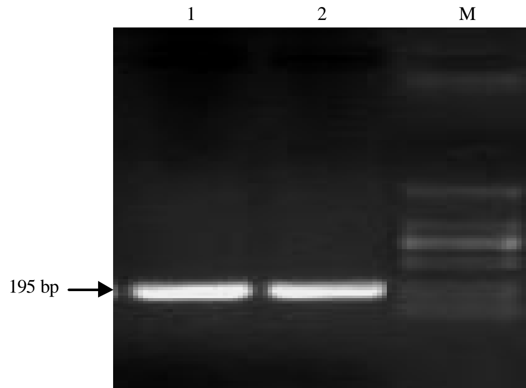

Fig. 2: PCR result of *Ler* gene from *E. coli*. M: DS2000 Marker

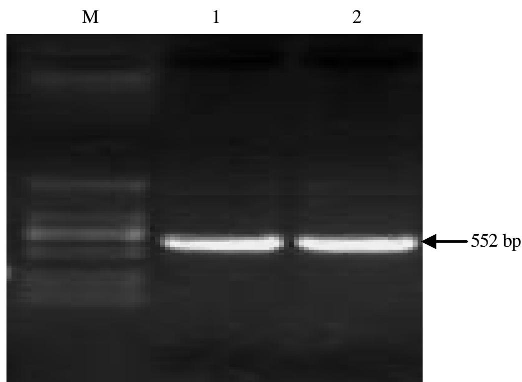

Fig. 3: PCR result of *eaeA* gene from *E. coli*. M: DS2000 Mark

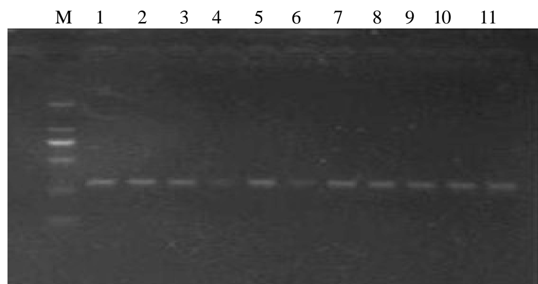

Fig. 4: PCR result of *irp2* gene from *E. coli* M: DM2000 Marker

Sequences homologous analysis of *irp2* gene: The 9 determined *E. coli* strains with *irp2* Virulence Island gene were retrieved by BLAST and then the sequences homologous comparison among 9 determined *E. coli* strains and referenced strains (um146, 10407, 83972, NA114 and O83H1) from GenBank was done by DNAstar. The results showed that sequence comparisons showed nucleotide identities were 99.6-100% among the tested *E. coli* strains and they shared the same identity to the referenced *E. coli* strains. The phylogenetic trees constructed from the *irp2* genes demonstrated that all *E. coli* strains were clustered into two branches. numbered 2, 4, 5, 11, 6 from tested strains and 83927, 10407, O83H1, NA114 from Genbank were clustered in the larger branches while numbered 3, 7, 10 and 16 strains were clustered in the other branch. The two branches showed nucleotide identities was 99.6% and each branch showed 100% which showed that there was no specific relations between serotype and irp2 Virulence Island, the homology was high among different serotype strains (Fig. 6 and Table 2).

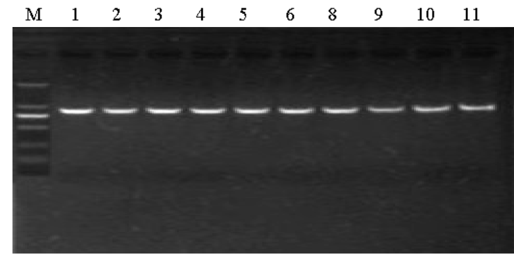

Fig. 5: PCR result of *fyuA* gene from *E. coli*. M: DM2000 Marker

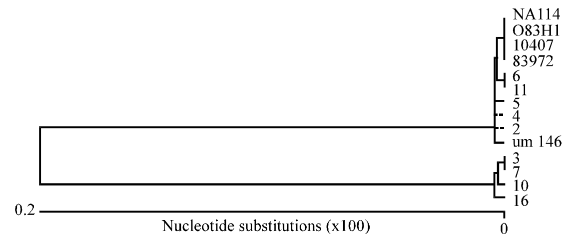

Fig. 6: The phylogenetic tree of nucleotide sequence of *irp2* genes

Sequences homologous analysis of *fyuA* gene: The 8 determined *E. coli* strains with fyuA Virulence Island gene were retrieved by BLAST and then the sequences homologous comparison among 8 determined *E. coli* strains and referenced strains (*um146*, 10393, *APEC01*, *fyuA* gene, *fyuA* and *S88*) from GenBank was done by DNA star. The results showed that sequence comparisons showed nucleotide identities were 99.8-100% among all of the *E. coli* strains. The phylogenetic trees constructed from the *fyuA* gene demonstrated that all *E. coli* strains were clustered into two branches. Numbered 11, 12, 1, 4, 5 and 6 from tested strains and *fyuA*, *S88*, *APEC01*, *fyuA* gene, um146 from Genbank were clustered in the larger branches while 7, 10 and 10393

Table 2: Sequences homologous analysis of *irp2* gene from *E. coli*

	Percentage identity													
Divergence	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	-	100.0	99.6	100.0	100.0	100.0	99.6	99.6	100.0	99.6	100.0	100.0	100.0	100.0
2	0.0	-	99.6	100.0	100.0	100.0	99.6	99.6	100.0	99.6	100.0	100.0	100.0	100.0
3	0.4	0.4	-	99.6	99.6	99.6	100.0	100.0	99.6	100.0	99.6	99.6	99.6	99.6
4	0.0	0.0	0.4	-	100.0	100.0	99.6	99.6	100.0	99.6	100.0	100.0	100.0	100.0
5	0.0	0.0	0.4	0.0	-	100.0	99.6	99.6	100.0	99.6	100.0	100.0	100.0	100.0
6	0.0	0.0	0.4	0.0	0.0	-	99.6	99.6	100.0	99.6	100.0	100.0	100.0	100.0
7	0.4	0.4	0.0	0.4	0.4	0.4	-	100.0	99.6	100.0	99.6	99.6	99.6	99.6
8	0.4	0.4	0.0	0.4	0.4	0.4	0.0	-	99.6	100.0	99.6	99.6	99.6	99.6
9	0.0	0.0	0.4	0.0	0.0	0.0	0.4	0.4	-	99.6	100.0	100.0	100.0	100.0
10	0.4	0.4	0.0	0.4	0.4	0.4	0.0	0.0	0.4	-	99.6	99.6	99.6	99.6
11	0.0	0.0	0.4	0.0	0.0	0.0	0.4	0.4	0.0	0.4	-	100.0	100.0	100.0
12	0.0	0.0	0.4	0.0	0.0	0.0	0.4	0.4	0.0	0.4	0.0	-	100.0	100.0
13	0.0	0.0	0.4	0.0	0.0	0.0	0.4	0.4	0.0	0.4	0.0	0.0	-	100.0
14	0.0	0.0	0.4	0.0	0.0	0.0	0.4	0.4	0.0	0.4	0.0	0.0	0.0	-

1: um146; 2: 2; 3: 3; 4: 4; 5: 5; 6: 8; 7: 7; 8: 10; 9: 11; 10: 18; 11: 10407; 12: 83972; 13: NA114; 14: O83H1

Table 3: Sequences homologous analysis of *fyuA* gene from *E. coli*

	Percent identity													
Divergence	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	-	100.0	100.0	100.0	100.0	99.8	99.8	100.0	100.0	99.8	100.0	100.0	100.0	100.0
2	0.0	-	100.0	100.0	100.0	99.8	99.8	100.0	100.0	99.8	100.0	100.0	100.0	100.0
3	0.0	0.0	-	100.0	100.0	99.8	99.8	100.0	100.0	99.8	100.0	100.0	100.0	100.0
4	0.0	0.0	0.0	-	100.0	99.8	99.8	100.0	100.0	99.8	100.0	100.0	100.0	100.0
5	0.0	0.0	0.0	0.0	-	99.8	99.8	100.0	100.0	99.8	100.0	100.0	100.0	100.0
6	0.2	0.2	0.2	0.2	0.2	-	100.0	99.8	99.8	100.0	99.8	99.8	99.8	99.8
7	0.2	0.2	0.2	0.2	0.2	0.0	-	99.8	99.8	100.0	99.8	99.8	99.8	99.8
8	0.0	0.0	0.0	0.0	0.0	0.2	0.2	-	100.0	99.8	100.0	100.0	100.0	100.0
9	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	-	99.8	100.0	100.0	100.0	100.0
10	0.2	0.2	0.2	0.2	0.2	0.0	0.0	0.2	0.2	-	99.8	99.8	99.8	99.8
11	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.0	0.2	-	100.0	100.0	100.0
12	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.0	0.2	0.0	-	100.0	100.0
13	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.0	0.2	0.0	0.0	-	100.0
14	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.0	0.2	0.0	0.0	0.0	-

1: um146; 2: 1; 3: 4; 4: 5; 5: 6; 6: 7; 7: 10; 8: 11; 9: 12; 10: 10393; 11: APEC01; 12: *fuya* gene; 13: fyua; 14: S88

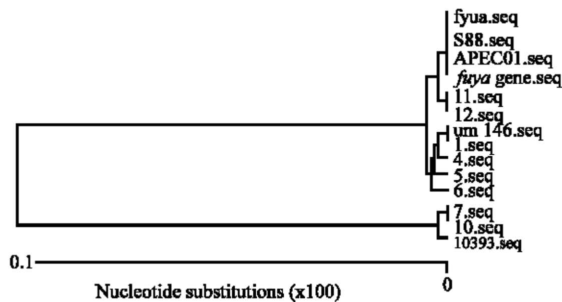

Fig. 7: The phylogenetic tree of nucleotide sequence of *fyuA* genes

strains were clustered in the other branch. The two branches showed nucleotide identities was 99.8% and each branch showed 100%. Which showed that there was no specific relations between serotype and fyuA Virulence Island, the homology was high among different serotype strains (Fig. 7 and Table 3).

Relations of *E. coli* serotype and Virulence Island: Identification of serotype was conducted according to the product instruction of China Institute of Veterinary Drug Control by Flat agglutinate experiment. The results showed that 11 were not serotyped and other 15 strains were clustered in eight serotypes: O91, O78, O107, O38, O88, O24, O9 and O11 which indicated that the pathogenicity of *E. coli* from chickens was complex in this area. The rates of O91, O38 and O78 strains were 20% (3/15), 20% (3/15) and 26.7% (4/15), respectively they were the dominant serotypes.

The total detective rates of *fyuA*, *irp2* and *irp2+fyuA+* gene were 42.3% (11/26), 73% (19/26), 42.3% (11/26), respectively. The rates of carrying HPI in the dominant serotypes O91, O38 and O78 were 7.7, 3.85 and 15.39%, respectively. While the rates in O107, O88, O24, O9 and O11 was 3.85% (O9 was 0). The carried rates of LEE from all strains were very low, only 1 strain was carried with *LEE* gene (Ler, eaeA), the rate was 3.85%. There was not *asn_RNA_intB* gene in all of the samples (Table 4).

Table 4: Relation of serotype and virulence island in 26 strains *E. coli*

Serotype count percent	Rate of LEE$^+$			Rate of HPI$^+$		
	ler$^+$	eaeA$^+$	ler$^+$eaeA$^+$	irp$^+$	fyuA$^+$	asn_RNA_intB$^+$
O91	3	20(3/15)	-	-	7.70	7.70
O78	3	20(3/15)	-	3.85%	3.85	3.85
O107	1	6.7(1/15)	-	-	3.85	0.00
O38	4	26.7(4/15)	-	-	15.39	11.54
O88	1	6.7(1/15)	-	-	3.85	0.00
O24	1	6.7(1/15)	-	-	3.85	3.85
O9	1	6.7(1/15)	-	-	0.00	0.00
O11	1	6.7(1/15)	-	-	3.85	3.85
LEE	11	42.3(11/26)	3.85%	-	30.77	15.39

DISCUSSION

O serotype of *E. coli*: *E. coli* is a conditional pathogenic bacteria, having complex serotypes. There are obvious distinctions among different animals, the same animals in different areas. The differences present the regional distribution and diversity. There were 26 *E. coli* strains from chickens, 11 were not serotyped and other 15 strains were clustered in eight serotypes: O91, O78, O107, O38, O88, O24, O9 and O11 which indicated that the pathogenicity of *E. coli* from chickens was complex in this area. The rates of O91, O38 and O78 strains were 20, 20 and 26.7%, respectively they were the dominant serotypes.

The results were consistent with some reports in parts of domestic. The O78, O88, O2, O45, O53 and O145 are the dominant serotype in Tianjin (Koczura *et al*., 2012; Ding and Yan, 2003); O78, O143, O15, O2, O88, O35 in Shandong (Wang *et al*., 2004); O141, O147, O149, O88, O78, O1, O2, O111, O115 in Henan (Wang and Tian, 2004); O78, O109 in Liaoning (Li *et al*., 2008); O120, O26, O70, O119, O141, O89, O137 in Sichuan; O18, O78, O15 in Guizhou.

There is a big differences in the dominant serotype reported from other provinces. Based on the above data, multiple serotypes exist in the same region, the local dominant serotype exist in most areas but some are alike, some different in distinct places.

Pathogenicity island: In the genus *Yersinia*, HPI exists in *Yersinia pestis*, *Yersinia eudotuberculosis* and *Yersinia enterocolitica* and is a larger chromosomal segment which determines Yersinia virulence or pathogenic levels. HPI contains the genes which can encode a highly efficient iron acquisition carrier, yersiniabactin (Ybt) and their regulatory genes and its main function is iron uptake and regulation. HPI is an essential genetic unit expresses of mice lethal phenotype (Chen *et al*., 2006). *Irp2* gene is the main structural genes of core conserved region of HPI and can be used as detection mark of HPI (Cheng *et al*., 2006). HPI of *E. coli* isolated from raccoon dog is rarely reported. Yersinia's HPI can been detected in rabbits, pigs, cows, chickens and cattle manure as well as pork, beef and hamburger patties (Ding and Yan, 2003) (Wang *et al*., 2004; Li *et al*., 2008).

The previous study was conducted by Wenjie *et al*. (2006), *APEC* gene in pathogenicity *E. coli* strains from chickens were detected in Jiangsu Provinces by PCR and spot hybridization there was a big distinction among the positive rates of different serotype carrying HPI Virulence Island. In the dominant serotypes including O78, O93, O88, O9, O86, O24 and O109, the highest rates of carrying HPI was 94.7% in O78, 50% in O24, 15.4, 27.3 and 12.5% in O93, O9, O109, respectively. But there was none in O86 and O88. In the non-dominant serotype strains there were high rates in O11, O2, O15, O74, etc. Makobe Ck discovered Locus of Enterocyte Effacement (LEE) 62.1% (41/66) and High Pathogenicity Island (HPI) 57.6% (38/66). The 6% (4/66) expressed only *fyuA* gene, 12.2% (8/66) irp2 only and 39.4% (26/66) expressed both *fyuA* and *irp2* genes (Makobe *et al*., 2012). In this study, the positive rates of tested strains carrying HPI in dominant serotypes including O91, O38 and O78 were 7.7, 3.85 and 15.39%, respectively. While in the non-dominant serotypes including O107, O88, O24, O9 and O11, the rate was 3.85% (O9 was 0). The rate of *irp2+fyuA+* gene was 42.3%, the rates of carrying LEE (ler, eaeA) in all strains were very low, the rate was 3.85%. There was not *asn_RNA_intB* gene in all of the samples. The above results was similar with Jinwenjie, etc.

CONCLUSION

Researchers can draw a conclusion from this study that there is a tendency of carrying HPI in different serotype strains. The reason is related with specified serotype but there is unobvious relations whether or not belongs to the dominant serotypes.

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