Genetic variations of avian *Pasteurella multocida* as demonstrated by 16S-23S rRNA gene sequences comparison

Jabbari, A. R.[1*]; Esmailzadeh, M.[2]; Moazeni Jula, G. R.[3] and Moosavi Shoushtari, M.[4]

[1]Pasteurella Research Laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran; [2]Department of Biotechnology, Razi Vaccine and Serum Research Institute, Karaj, Iran; [3]Department of Aerobic Bacterial Vaccines, Razi Vaccine and Serum Research Institute, Karaj, Iran; [4]Department of Anaerobic Bacterial Vaccines, Razi Vaccine and Serum Research Institute, Karaj, Iran

[*]**Correspondence:** A. R. Jabbari, Pasteurella Research Laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran. E-mail: a.jabbari@rvsri.ir

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Summary

Pasteurella multocida is known as an important heterogenic bacterial agent causes some severe diseases such as fowl cholera in poultry and haemorrhagic septicaemia in cattle and buffalo. A polymerase chain reaction (PCR) assay was developed using primers derived from conserved part of 16S-23S rRNA gene. The PCR amplified a fragment size of 0.7 kb using DNA from nine avian *P. multocida* isolates. Sequence alignment of the 16S-23S rRNA genes (ITS) revealed a considerable heterogenicity among the isolates. The percentage of similarity varied from 83.3 to 100% among the isolates. An interesting finding from this study was the presence of an inserted sequence (seven nucleotides) in the 16S-23S rRNA region in 55% of the isolates. According to phylogenic analysis based on ITS sequence alignment, the *P. multocida* isolates classified into 2 distinct clusters. The virulence of isolates in cluster II were higher than those in cluster I. Ribotyping of *P. multocida* by using 16S-23S rRNA gene PCR sequencing could be used as a marker in epidemiologic studies.

Key words: *Pasteurella multocida*, ITS sequence, Ribotyping

Introduction

Pasteurella multocida has been recognized as an important animal pathogen, responsible for fowl cholera in chickens and turkeys, haemorrhagic septicaemia and shipping fever in cattle, atrophic rhinitis in piglets and snuffle in rabbits (Rimler and Rhoades, 1989).

Fowl cholera (avian pasteurellosis) is a contagious septicaemic disease, usually associated with high mortality and morbidity in chickens, turkeys, ducks and numerous other domestic and wild birds. Outbreaks of fowl cholera happen in peracute, acute or chronic forms (Rimler and Gilson, 1997). *P. multocida*, the causative agent, is classified by different conventional and molecular methods. The conventional methods for the identification of a suspect isolate as *P. multocida* involve subjecting the isolate to a range of bacteriological and biochemical tests (Rimler and Rhoades, 1989).

The rRNA genetic loci contain the genes for 16S and 23S fragments. These genes are separated by a spacer region which exhibits a large degree of sequence and length variation at the level of genus and species (Jensen *et al*., 1993). The 16S-23S rRNA spacer region is transcribed together with the ribosomal genes and thus is named an internal transcribed spacer (ITS) (Gurtler and Stanisich, 1996).

The most direct and rapid method to visualize the polymorphic character of 16S-23S rRNA spacer is to carry out PCR amplification of the spacer regions by using primers from highly conserved flanking sequences (Jensen *et al*., 1993). PCR ribotyping of ITS has been used to

differentiate strains of bacteria such as *Staphylococcus aureus* on the basis of sequence variations of the region (Kostman *et al*., 1995). In the present study we explored the natural polymorphism of the genomic 16S-23S rRNA region from *P. multocida* as a genotyping tool.

Materials and Methods

Bacterial strains

A total of nine isolates of *P. multocida* used for this study are described in Table 1. All isolates were obtained from poultry cases whose death was due to fowl cholera in Northern provinces of Iran (Gilan and Mazandaran). The isolates were kept at -70°C since the original isolation or as freeze-dried cultures. Ampoules containing freeze-dried cultures were opened under sterile conditions, and the content was dissolved in brain heart infusion broth (Difco), subsequently inoculated on blood agar (containing 5% sheep blood) and incubated at 37°C for 24 h, to ensure that they represented pure cultures.

Table 1: *Pasteurella multocida* isolates and their related ITS sequence accession numbers in the GenBank

Isolate	Origin	Accession number
PMI30	Duck-Gilan	DQ157903
PMI22	Chicken-Mazandaran	DQ157898
PMI03	Chicken-Mazandaran	DQ157899
PMI04	Chicken-Mazandaran	DQ157900
PMI05	Chicken-Mazandaran	DQ157901
PMI20	Chicken-Mazandaran	DQ157902
PMI32	Chicken-Mazandaran	DQ157904
PMI46	Chicken-Mazandaran	DQ157905
PMI47	Chicken-Mazandaran	DQ157906
PM70	Avian-United States	AE006209

Phenotypic characterization

Phenotypic characterization was done according to Bisgaard *et al*. (1991) to allow classification into one of three subspecies of *P. multocida* (*multocida*, *septica*, *gallicida*). The capsular typing of the isolates was determined by the PCR method of Townsend *et al*. (2001).

DNA extraction

A 1 µl sterile disposable loop was used to lift a small amount of colony material from the surface of an agar plate into 200 µl sterile phosphate buffered saline (PBS, pH = 7.2) in a 0.5 ml tube. After vortexing, the suspension was heated on a thermal cycler at 98°C for 15 min. The cell debris was pelleted by centrifugation at 13000 g in a benchtop microfuge for 5 min and 1 µl of the supernatant was used as DNA template.

16S-23S PCR

For PCR amplification, the following primers were used: 16S-23S FW: 5'-TTG,TAG,ACA,CCG,CCC,GTC,A-3' and 16S-23S RV: 5'-GGT,ACG,TTA,GAT, GTT,TCA,GTT,C-3'. The primers were designed corresponding to rRNA gene of *P. multocida* by using the sequence data obtained from GenBank (National Institutes of Health). The reactions were performed in a final volume of 50 µl at the following reagent concentrations: 10 mM Tris-HCl, pH = 8.3, 50 mM KCl, 200 µM dNTP, 0.5 µM of each primer, 3 mM $MgCl_2$, 2.5 U Taq polymerase enzyme, and 1 µl of template DNA. Amplification mixtures were submitted to 40 cycles: 94°C for 1 min, 72°C for 2 min, and final extension at 72°C for 7 min. PCR products were detected by horizontal gel electrophoresis of 10 µl aliquot in a 1.5% agarose gel containing ethidium bromide in TAE buffer and visualized under UV light. Molecular sizes were determined based on a 100 bp ladder molecular weight marker. All amplifications were performed with the Eppendorf PCR system.

Sequencing

PCR products for sequencing were purified using the PCR product purification kit (Roche, Germany). All purified PCR products were sequenced by MWG Laboratory, Germany.

GenBank accession numbers

The ITS sequences of Iranian *P. multocida* isolates has been submitted to the GenBank. The GenBank accession numbers for 16S-23S spacer region nucleotide sequences determined in this investigation are listed in Table 1.

Analysis of the sequence data

Searches for sequences in GenBank

databases were performed by Blast. The comparison of the sequence alignments was done by Megalign software. The alignments of Iranian isolates were compared with each other and with the ITS sequence of American reference strain PM70 with accession number of AE0062091.

Results

Biochemical characterization and capsular typing showed that all the isolates of *P. multocida* belonged to subspecies multocida and capsular group A.

PCR amplification of 16S-23S rRNA gene of the isolates produced a single fragment of 0.7 kb (Fig. 1).

Partial sequences of the fragment were aligned using Megalign software.

Fig. 2 shows the alignment of the 16S-23S rRNA spacer sequences of nine Iranian *P. multocida* comparing with American strain PM70, the only available sequence in the GenBank (so far).

Nucleotide alignment showed an inserted sequence (IS) which was repeated among 5 (55%) of the Iranian isolates together with the American reference strain PM70. It contained seven nucleotides including TAATCAA. The position of IS in two isolates (PMI03 and PMI47) was the same as strain PM70 at nt 8261. This site for three other isolates (PMI05, PMI22, PMI46) was at nt 8268.

ITS sequences of four isolates (PMI04, PMI20, PMI30 and PM32) did not consist of the above inserted sequence.

According to phylogenetic analysis, the investigated isolates were classified into 2 main clusters (Fig. 3). Cluster I included all five *P. multocida* isolates which had the IS in their ITS region. *P. multocida* isolates without inserted sequence (four remain isolates) belonged to cluster II.

As it is presented in Table 2 the similarity of the ITS among the isolates ranged from 83.3 (between isolates PMI20 and PMI22) to 100% (between PMI03 and PMI05).

Restriction map analysis showed the presence of a specific restriction site of H*pa*I at the position of IS among *P. multocida* isolates in cluster I.

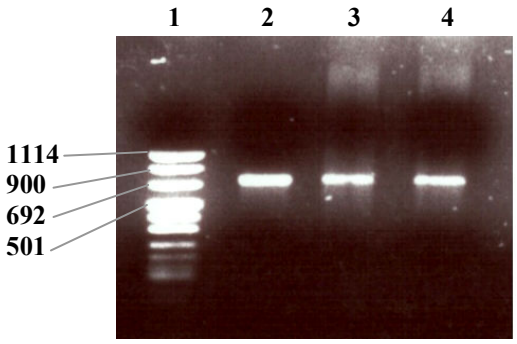

Fig. 1: PCR amplification of 16S-23S rRNA gene of the isolates produced 0.7 kb fragment. Lane 1: DNA size marker and Lanes 2-4: the PCR products with the DNA from *P. multocida* isolates: PMI30, PMI04 and PMI20, respectively

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AE0062091 8198  AACCATACAACCTCAAGTATTCTTACTTAATCTCAATTAATCCATTAACCTTTGCTAATG  8257
DQ157903  494   ............................................................  435
DQ157900  481   ............................................................  422
DQ157902  497   ...........................................C................  438
DQ157901  503   ..........................C.............TT...........T...CC  444
DQ157904  427   ............................................................  368
DQ157898  449   ..........................C.........................T...CC  390
DQ157905  449   ..........................C............T......T....T...CC  390
DQ157906  168   ..........................C............T......T....T...CC  109
DQ157899  141   ..........................C............TT...........T...CC  82

AE0062091 8258  TAT-------TAATCAA-------GTTTCATCAACTCGATGTTGTGTTTAAACGCTAAAC  8303
DQ157903  434   C..-------.......-------.........C..T.......................  389
DQ157900  421   C..-------.......-------.........C..T.......................  376
DQ157902  437   C..-------.......-------.C.......C..T.......................  392
DQ157901  443   ...-------.......TAATCAA.C..........T....C..................  391
DQ157904  367   C..-------.......-------.....................................  322
DQ157898  389   ...-------.......TAATCAA.C..........T....C..................  337
DQ157905  389   ...-------.......TAATCAA.C..........T....C..................  337
DQ157906  108   ...TAATCAA.......-------.C..........T....C..................  56
DQ157899  81    ...TAATCAA.......-------.C..........T....C..................  29
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Fig. 2: Alignment of the 16S-23S rRNA spacer (ITS) sequences (partial) of Iranian avian *P. multocida* comparing with American strain PM70

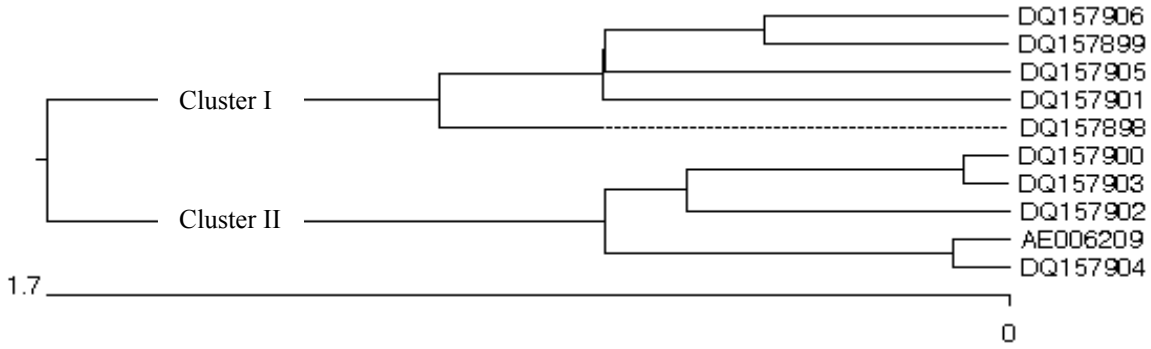

Fig. 3: Phylogenic relationship dendrogram of avian *Pasteurella multocida* isolates according to 16S-23S region sequence

Table 2: Percent of identity and divergence according to ITS region sequences of avian *Pasteurella multocida* isolates

Percent identity

Divergence	1	2	3	4	5	6	7	8	9	10		
1		91.7	99.2	99.2	91.3	99.2	90.6	91.3	91.3	98.5	1	DQ157906
2	4.0		93.9	90.7	97.8	95.8	97.5	96.2	99.8	95.2	2	AE006209
3	0.8	2.0		99.2	93.0	92.7	88.3	92.1	94.1	96.1	3	DQ157898
4	0.9	4.5	0.9		90.3	100.0	90.3	90.3	90.3	97.9	4	DQ157899
5	4.4	1.3	2.9	5.0		92.7	96.1	99.0	98.9	95.4	5	DQ157900
6	0.8	1.9	0.7	0.0	2.8		96.1	93.8	96.6	99.1	6	DQ157901
7	4.8	1.9	2.9	5.0	1.2	2.8		97.5	97.7	95.4	7	DQ157902
8	4.4	1.3	2.9	5.0	0.2	2.8	1.0		97.9	94.5	8	DQ157903
9	4.4	0.2	2.3	5.0	1.0	2.3	1.7	1.0		95.2	9	DQ157904
10	0.0	2.5	1.1	0.9	2.5	0.7	2.5	2.5	2.8		10	DQ157905
	1	2	3	4	5	6	7	8	9	10		

Discussion

PCR ribotyping is a fast and reliable typing method with good typing ability and reproducibility for several bacterial species, such as *S. aureus*, *E. coli* and *Enterobacter* sp. This approach targets the 16S-23S rRNA region which is polymorphic and repetitive in the genome of such bacteria (Oliveira and Ramos, 2002).

Sequence analysis of ITS following PCR amplification has been used for investigation in genetic diversity of some bacteria.

The length of ITS region located between 16S and 23S rRNA gene shows a wide variation both among species and among different copies of the ribosomal operon within a chromosome. Osorio *et al*. (2005) demonstrated the genetic diversity not only between subspecies but also between strains of the same subspecies by ITS sequence analysis. They found that these length variations in ITS region are due, mainly, to the type and number of tRNA gene interspersed (Osorio *et al*., 2005).

In the present study the sequence variations in the 16S-23S rRNA genes of nine local *P. multocida* isolates were investigated. The similarity ranged from 83.3 (between PMI20 and PMI22) to 100% (between PMI05 and PMI03). As it was shown in Fig. 2, another finding was an inserted sequence (IS) of seven nucleotides which was at positions 8261 (for two isolates) and 8268 (for three isolates).

Biochemical characterization and capsular typing were not able to discriminate the isolates. As demonstrated in the present investigation, the genetic diversity of organisms causing fowl cholera is considerable. Previous investigations have also shown that *P. multocida* associated with fowl cholera represented multiple clones (Petersen *et al*., 2001). Fussing *et al*. (1999) found high genotypic diversity among toxin-producing *P. multocida* isolates. They divided 68 *P. multocida* isolates into 18 different ribotypes. Petersen *et al*. (2001) found at least six ribotype clusters among avian isolates of *P. multocida*.

Christensen *et al*. (2004) used ribotyping as a tool for classification of atypical isolates

of *P. multocida* from bovine lungs. ITS fragment-length profiling showed identity of the majority of strains (47 of 52), with only five divergent strains. Davies (2004) investigated the genetic diversity among *P. multocida* isolates from avian, bovine, ovine and porcine origin by sequence analysis of the 16S rRNA gene. Nineteen 16S rRNA types were identified, but these were clustered into two distinct phylogenetic lineages, A and B.

Preliminary investigations on virulence of the isolates have shown that the pathogenicity of isolates in cluster II was higher than isolates in cluster I. However, because of virulence complexity in *P. multocida* the complementary studies are needed to show the exact relationship.

According to the findings of this study, considerable polymorphisms were shown in the 16S-23S rRNA gene of *P. multocida*. Another interesting finding was the repeated sequence which classified the isolates into 2 clusters and could be used as a marker in epidemiologic studies.

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