Characterization of Avian *Pasteurella multocida* Isolates by Protein Profiles and Restriction Enzyme Analysis of Chromosomal DNA

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Received 14 May 2002; accepted 11 Aug 2002

Summary

The protein profiles of 25 avian *Pasteurella multocida* isolates were studied by discontinuous polyacrylamide gel electrophoresis. The Coomassie blue stained gel showed that each whole cell protein lysate of *P multocida* contained over 30 protein bands ranging from 24 to 174 KDa. Majority of protein bands was located in the mid section of the gel between 30 and 97 KDa. All isolates were similar in the majority of protein bands. The major difference between protein patterns of isolates was revealed in the position of a major outer membrane protein band (H Protein) presented in the 34-38 KDa regions. According to molecular mass, the isolates were classified in three protein profiles. Restriction enzyme analysis (REA) of chromosomal DNA by *HpaII* distinguished six groups, two of which contained a single isolate. The Iranian vaccine strain (PMI030) and thirteen field isolates belonged to REA type I. There was not any relationship between serotype, protein profile and REA pattern of the isolates.

Key words: *Pasteurella multocida,* poultry, protein profile, restriction enzyme analysis

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Introduction

Fowl cholera is a septicaemic disease of chickens, turkeys, ducks and waterfowl and caused by certain strains of *Pasteurella multocida* (Diallo *et al* 1995). *P.multocida* has been extensively studied since it was first isolated in the late 1870s (Rhoades & Rimler 1991). Fowl cholera is known as one of the most serious death and economic losses in northern parts of Iran (Kalaydari 1998, Sotoodehnia *et al* 1986, Bozorgmehri fard & Afnan 1972). In recent years, the application of new technologies has improved the knowledge on the organisms. Polyacrylamide gel electrophoresis (PAGE) of proteins has been used increasingly during the past decade in many bacterial classification and identification (Bahaman *et al* 1994, Johnson *et al* 1991, Adam *et al* 1991) and to identify protective antigens (Ireland *et al* 1991). This technique is simple to perform with large numbers of samples, and to obtain great information about bacterial isolates (Lema & Brown 1983). Restriction endonuclease analysis (REA) has been proved valuable for discrimination of bacterial isolates, and identification of unique strains within groups of related organisms (Kim & Nagaraja 1990, Draual *et al* 1992, Wilson *et al* 1995, Smart *et al* 1988). Restriction endonucleases cleave the DNA at specific nucleotide sequences and produce a set of DNA fragments which, when separated by electrophoresis provide a characteristic band pattern or fingerprint of the respective genome. The technique has already been used in molecular studies of field isolates of *P.multocida* from haemorrhagic septicaemia (Wilson *et al* 1995), fowl cholera (Diallo *et al* 1995, Kim & Negaraja 1990, Carpenter *et al* 1991, Christiansen *et al* 1992, Blackal *et al* 1995) and atrophic rhinitis (Harel *et al* 1990). The objectives of present study were to characterize the protein and DNA fingerprints profiles of *P.multocida* isolates obtained from poultry in Iran.

Materials and Methods

Bacterial isolates and culturing method. The *P.multocida* isolates that were examined in this study are shown in Table 1. The lyophilized form of the isolates

were resuspended in 1ml brain heart infusion (BHI) broth and incubated at 37°C for 1h. A loopful of bacterial suspension was cultured onto 5% sheep blood agar plates and incubated at 37°C for 24h. For protein preparation the plates were washed by 2ml phosphate buffer saline (PBS) pH7.4. The washing buffer was removed by centrifugation at 13000*g* for 5min. For DNA extraction growth from a single colony was inoculated into a test tube containing 3ml BHI broth and incubated at 37°C for 18-24h with a little rotation. 1.5ml aliquots of the BHI were centrifuged at 13000*g* for 5min. The pellets were washed by PBS and used for DNA preparation. The serotypes of the isolates were studied by Hedleston system previously (Jabbari *et al* 2001).

Protein preparation. Whole cell (WC) protein was prepared by adding sample buffer to the *P.multocida* pellet. The sample buffer consisted of 0.1M Tris HCl (pH6.8), 15% glycerol, and 2% sodium dodecyl sulphate (SDS). The pellets were resuspended in sample buffer by vigorous vortexing. The mixed cells were heated in a water bath for 5min at 95°C. The microtubes of WC soluble protein were stored at –20°C until use.

Polyacrylamide gel electrophoresis. The discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970). Electrophoresis in a 4% stacking gel and 12% resolving gel was performed using a vertical protein system. Polymerisation was initiated by adding 10µl tetrametylendiamine (TEMED). Following polymerisation of the gel, the comb was removed and the wells were rinsed with distilled water. The gel was clipped onto the electrodes unit of the apparatus. The running buffer (Tris-glycerin) was poured into the chamber of the apparatusand the samples (25-30µg protein) loaded in the wells. Electrophoresis was carried out at 50V until the tracking dye reached the resolving gel. The voltage then increased to 120V and electrophoresis continued until the dye reached 1cm from the bottom of the gel. The gel was stained with 0.3% Coomassie blue solution. It was destained using a solution containing 10% glacial acetic acid until the background was clear.

Table 1. *Origins of twenty-five P.multocida isolates obtained from poultry in northern part of Iran*

Study code	Origine	Province	Year	Source
PMI032	Chicken	Mazandara	1997	Present study
PMI033	Duck	Mazandara	1997	Present study
PMI034	Chicken	Gilan	1997	Present study
PMI035	Duck	Mazandara	1997	Present study
PMI036	Geese	Mazandara	1997	Present study
PMI037	Duck	Gilan	1997	Present study
PMI038	Geese	Mazandara	1997	Present study
PMI039	Duck	Mazandara	1997	Present study
PMI040	Chicken	Mazandara	1998	Present sudy
PMI041	Chicken	Mazandara	1998	Present study
PMI042	Chicken	Mazandara	1987	RVSRI*
PMI043	Chicken	Mazandara	1997	RVSRI
PMI044	Duck	Mazandara	1997	RVSRI
PMI045	Duck	Mazandara	1997	RVSRI
PMI046	Chicken	Mazandaran	1997	RVSRI
PMI047	Chicken	Mazandaran	1998	Present study
PMI022	Chicken	Mazandaran	1998	Present study
PMI023	Chicken	Gilan	1997	Present study
PMI024	Chicken	Gilan	1997	Present study
PMI025	Chicken	Gilan	1994	RVSRI
PMI026	Chicken	Mazandaran	1994	RVSRI
PMI028	Chicken	Mazandaran	1994	RVSRI
PMI030	Chicken	Gilan	1981	RVSRI
PMI031	Duck	Gilan	1994	RVSRI
PMI020	Chicken	Mazandaran	1994	

*RVSRI: Razi Vaccine & Serum Research Institue

Preparation of whole cell DNA. Genomic DNA from 25 *P.multocida* isolates was prepared according to Wilson *et al* (1995) with some modifications. Briefly, bacterial cells were lysed by EDTA, SDS and proteinase K. The DNA was precipitated with one volume of pure phenol saturated with Tris pH8.3. The upper

phase was collected and extraction was repeated two times more with one volume of phenol choloroform isoamyl alcohol mixture (24:24:1). The DNA was precipitated by the addition of sodium acetate and absolute ethanol. The DNA was then washed with 70% ethanol, dried at room temperature and resuspended in TE (Tris EDETA) buffer (pH 8).

Restriction enzyme digestion of a genomic DNA. Approximately 1 to 2µg of DNA sample was used for each restriction endonuclease digestion. Prelimnary studies for restriction endnuclease were done with *EcoRI, HpaII, Hind III* and *CfoI*, of these *HpaII* yielded fingerprint profiles that were best distinguished. Therefore, all digestions were conducted with this endonuclease. Digestion of the chromosomal DNA was done with 10U/µl of each endonuclease in a 30µl volume. The DNA mixture containing the DNA sample as substrate, restriction enzyme (10U), 1×reaction buffer and deionized distilled buffer was incubated at 37°C in a water bath for 5-6h. The digested DNA was mixed with loading buffer and electrophorese in 0.8% agarose at 25V for 17h. The gels were stained in ethidium bromide (0.25µg/ml) for 30min and visualized by UV transluminator. The recognition sequence of the mentioned enzymes are as follows: *EcoRI* (G/AATTC), *Hind III* (A/AGCTT), *CfoI* (G/ANTC) and *HpaII* (C/CGG) (Towner & Cockayne 1993).

Results

Electrophoretic protein patterns of isolates. Upon examination of the Coomassie blue stained gels, it was found that each of bacterial lysates contained over 30 polypeptide bands, covering an estimated molecular weight ranging from 24 to 174 KDa (Figure 1). However, the majority of protein bands were located in the mid section of the gel between 30 and 97 KDa. Although the overall protein profiles were usually similar, there were some clear differences between protein profiles of strains examined by SDS-PAGE. All isolates were similar in the majority of protein bands. The major difference between protein patterns of isolates was revealed in the position of one of the major protein (H protein) presented in the 34-38 KDa regions.

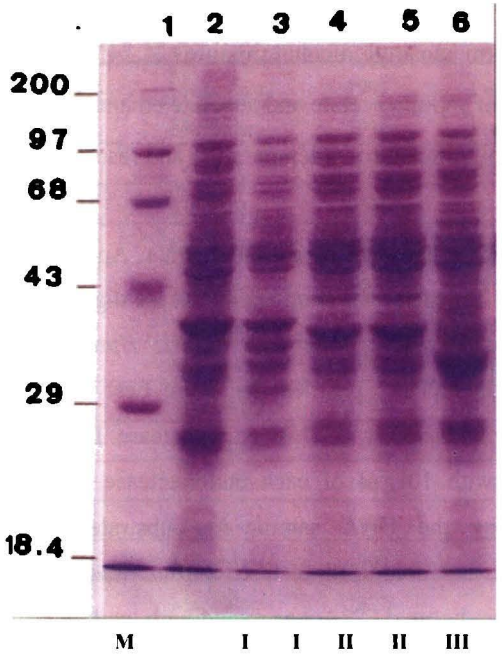

Figure 1. *SDS-PAGE whole cell protein fingerprinting of representative P.multocida isolates. Lanes 1-6 contain the Protein Marker (M), PMI030, PMI028, PMI045, PMI046 and PMI020. The arrow points to the H protein band position. The protein types of the isolates are presented at the bottom of lanes*

Based on H protein position, 3 distinguishable groups were identified (Table 2). In protein pattern type I, the molecular mass of H protein was about 38 KDa but in protein patterns type II and III, the molecular weight of this protein was 36.5 and 34 KDa, respectively (Figure 1).

Restriction enzyme analysis (REA) of chromosomal DNA. All isolates of *P.multocida* were subjected to REA with *HpaII* digestion. Isolates with matching *HpaI*I REA patters were grouped based on visual analysis of the initial gels. To verify the REA patterns of the isolates, restriction endonuclease digestion with *HpaII* and electrophoresis was repeated three times for each isolate. Isolates classified as the same *HpaII* REA type have restriction patterns that matched on number and position of all fragments heavier than 6.1Kb. It was the zone over which

fragments were clearly defined. However, the enzyme *HpaII* gave clears patterns that allowed the 25 isolates to be divided in 6 groups, 2 of which contained a single isolate (Table and figure 2).

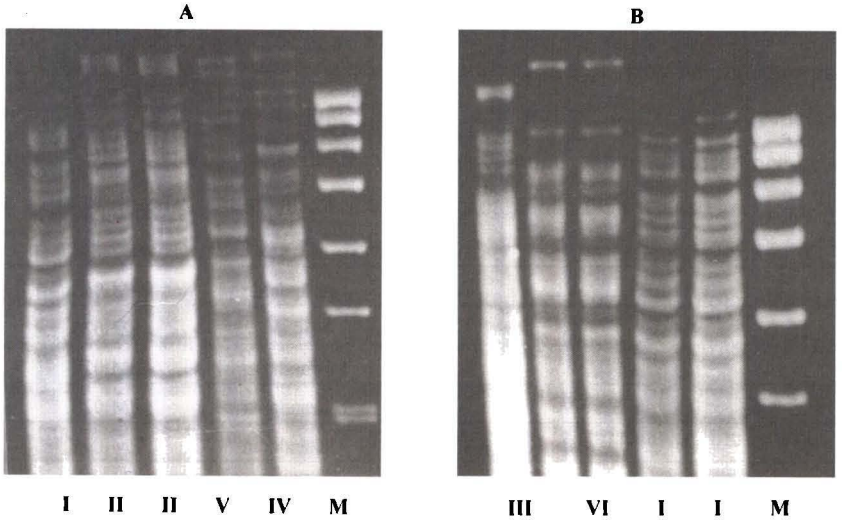

Figure 2: *HpaII restriction fragment patterns of chromosomal DNA of representative P.multocida isolates. Gel A, lanes 1-6 contain PMI020, PMI047, PMI022, PMI028, PMI025 and the DNA Marker (SPP1 DNA cleaved with EcoR1). Gel B, lanes 1-6 contain PMI026, PMI045, PMI046, PMI044, PMI034 and the DNA Marker. The REA types are presented at the bottom of the lanes*

Among the isolates from Gilan province 3 REA types, types I, III and IV, were identified. Four of five *P.multocida* isolates with REA type I (PMI023, PMI024, PMI030 and PMI034) and type IV (PMI025) were obtained from chicken while the isolate with type IV (PMI031) was originated from a duck in Gilan province. Five different REA fingerprint types were observed within *P.multocida* isolated from poultry in Mazandaran province. They were REA types I, II, III, V and VI. REA types IV and I were identified in both provinces. Whereas REA types II, V and VI were observed only among *P.multocida* strains obtained from Mazandaran province.

Discussion

Characterization of protein profiles of many bacterial species has been studied by

Table2. *Serotypes, Protein patterns, REA types and designated DIE code of 25 avian Pasteurella multocida isolated from Iran*

Serotype	Protein Type	REA Type	DIE*	Province	Isolates
1	I	I	-:1,I,I	Gilan Mazandaran	PMI030-PMI034-PMI037-PMI023-PMI024 PMI033-PMI036-PMI038-PMI039-PMI040-PMI041-PMI042-PMI043
1	II	VI	-:1.II,VI	Mazandaran	PMI045
1	II	IV	-:1,II,IV	Gilan	PMI025
2	III	I	-:2,III,I	Mazandaran	PMI020
2	I	V	-:2,I,V	Mazandaran	PMI028
3	II	II	-:3,II,II	Mazandaran	PMI035
3	II	VI	-:3,II,VI	Mazandaran	PMI046
3	III	II	-:3,III.II	Mazandaran	PMI022
4	III	II	-:4,III,II	Mazandaran	PMI047
3x4	I	III	-:3x4,I,III	Gilan	PMI031
3x4	II	II	-:3x4,II,II	Mazandaran	PMI032
3x4	II	III	-:3x4,II,III	Mazandaran	PMI026

*Descriptive identification epithet code

using SDS-PAGE (Achtman *et al* 1991, Adam *et al* 1991, Verstrate & Winter 1984, Jackman & Peleczynska, 1986). The protein profiles of Pasteurella genus have been also studied by SDS-PAGE. Some investigators focused on WC bacterial lysate (Bahaman *et al* 1994, Johnson *et al* 1991, Lugtenberg *et al* 1984, Ireland *et al* 1991) and others studied outer membrane protein extract (Choi *et al* 1989, Lu *et al* 1991, Vasfi Marandi & Mittal 1995, Ramandi & Adler 1994). In this study the protein profiles of *P.multocida* obtained from poultry were characterized. It was demonstrated that there are at least 8 similar protein bands in the protein profiles of the isolates ranging from 27 to 97 KDa molecular weight. Results of this study showed that SDS-PAGE protein pattern type I was dominant among isolates from

Gilan province. There was no isolate belonged to type III in this area. Among isolates obtained from Mazandaran province 58% of isolates belonged to protein pattern type I, 23.5% was type II and 17.6% was identified as type III. These results showed that all protein type III isolates were obtained from poultry in Mazandaran area. About 64% of *P.multocida* isolates examined in this study belonged to protein fingerprints type I, types II and III with 24% and 12% frequency respectively. Although the majority of protein bands were similar, the main difference between the isolates was in the position of a major protein band known as H protein, which was present in the 34-38 KDa regions. These results were in agreement with the finding of Ireland *et al* (1991). They demonstrated that the related molecular mass of H protein in two clinical isolates and Hedleston serotype one strain was 38 KDa, one isolate 36 KDa and 8 other clinical isolates 34 KDa. In the present study we classified isolates with 38 KDa H protein in group I (16 isolates), the isolates with 36.5 KDa H protein in group II (6 strains) and 3 other isolates having 34 KDa H protein, in group III.

According to the findings of this research, there was not any serotype specific protein pattern among the isolates. However 14 from 16 (87.5%) isolates of serotype 1 belonged to protein profile type I. This finding is supported by Choi *et al* (1989) in which they did not find any serotype specific protein marker among the reference strains of *P.multocida*. Genomic analysis of 25 isolates of avian *P.multocida* strains obtained from various hosts and geographic areas demonstrated that there was a considerable genetic diversity among the strains. REA using *HpaII* was useful in detecting differences in the *P.multocida* isolates that could not be distinguished by conventional phenotypic methods. A specific serotype was consisted of more than one genomic fingerprint. For example the most common serotype (serotype 1) consisted of three different DNA patterns in the present study (Table 2).

Some questions were raised regarding the relationship between phenotypic characters (such as serotype and protein pattern) and REA genotypic characters. Because there were isolates with the same REA type but different phenotypes, as

well as isolates with different REA types that showed the same phenotype. It would be possible for different genotypes to contain portions of their genomes that encode certain similar antigens genes such as serotype determinant antigens or H protein but have significant other portions of the genome that are different. Additionally, phenotypic characteristics could be under the control of environmental influences, many of which are unknown. A good example of phenotypic variation under the influence of the external environment is the iron -regulated outer membrane proteins of *P.multocida* (Snipes *et al* 1988). The situation in which isolates had the same REA type but different phenotype can be explained by the fact that the sequence of nucleotides genes encoding phenotypic antigens such as LPS or H protein in the fragments of DNA can have a light difference whereas the total molecular weight of the band is not changed.

The results of this study were in general agreement with the results of other investigators. Snipes *et al* (1989) classified 25 isolates of *P.multocida* obtained from turkeys in to 7 REA groups. Blackall *et al* (1995) have shown advantages of using *HpaII* to produce restriction fragment length polymorphism patterns for Australian *P.multocida* strains. They found the technique to be very useful in establishing among their isolates subdivisions that were in absolute agreement with those derived from ribotyping. Diallo *et al* (1995) classified 39 strains of *P.multocida* isolated from turkey in eastern Australia within 10 groups, 3 of which contained a single isolate. This study showed that 14 isolates of *P.multocida* had similar genotype (REA) and phenotype (serotype and protein pattern) characteristics. It is possible that these isolates maybe have originated from a common colony source (Table 2). REA of DNA could reveal the genetic diversity of isolates in one district. For example 3 different REA types within Gilan isolates (I, III, and IV), 5 REA types (I, II, III, V, VI) from Mazandaran isolates were determined.

A descriptive identification epithet (DIE) code consisted of serologic characteristics and fingerprint profile designation with one or more endonucleases

has been introduced by Wilson *et al* (1995). According to the results of the present study and findings of our previous study (Jabbari *et al* 2001), a DIE consisting of serologic identification, protein pattern and REA fingerprint profile of DNA with *HpaII* combination is suggested. Therefore the characteristics of an isolate can describe phenotypically and genotypically. For example DIE of vaccine strain (PMI030), was designed as:1, Pr I, REA I. It was found that 13 other isolates had the same DIE as vaccinal strain (Table 2). This combination code is a suitable tool for epidemiologic study of *P.multocida* infections. In general, the results of this study showed that protein and DNA fingerprinting are useful in strain differentiation of *Pasteurella multocida* isolates obtained from poultry. It seems that a combination of phenotypic and genotypic characters of the isolates, make a more precise identification tool in order to select vaccine strains.

Acknowledgements

The authors are grateful to the support of the Razi Vaccine & serum Research Institute. Thanks also to Dr. A.Sotoodehnia and Dr. J.V Yoosefi for their cooperation. The Iranian Ministry of Jahad-e-Agriculture supported this work.

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