Molecular Typing of Avian *Pasteurella multocida I*solates by PCR-RFLP of *ompH G*ene

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Abstract
Molecular typing of twenty-five Pasteurella multocida isolates has been assessed by restriction fragment length polymorphism (RFLP) of a species-specific PCR assay. Amplification was based on the gene ompH, encoding a major outer memberane protein. RFLP analysis of the 1.2 kb ompH-amplification using EcoRI, HindIII and CfoI endonucleases produced 7 different patterns for the twenty five isolates of the four P.multocida serotypes. The PCR-RFLP of the ompH gene was found to be potentially a useful method for typing of P. multocida and therefore, for studying the epidemiology of P. multocida infections.
Keywords*: Pasteurella multocida; RFLP-PCR Typing; Protein H.*

INTRODUCTION

P. multocida is a non-motile, small Gram-negative rod of the family pasteurellaceae. It 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. Its importance as a human pathogen has increasingly been recognized in the last 50 years (Rimler and Rhoades, 1989).

The classification of *P. multocida* is traditionally based on immunological methods. Capsular typing, somatic serotyping and subserotyping are based respectively on the immunospecificity of the capsule antigens, lipopolysaccharide and a major outer membrane protein , OMPH (Carter, 1955; Hedleston *et al*., 1972; Vasfi Marandi and Mittal., 1997).

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The use of molecular methods, such as restriction endonuclease analysis of chromosomal DNA and ribotyping has provided considerable information about genomic characteristics of the organism (Townsend *et al*., 1997; Jabbari *et al*., 2002a). A limitation of both methods is that they are time consuming. For example it needs a long time for digestion, electrophoresis, blotting and hybridization in the case of ribotyping. Among different molecular methods which have been explained for genomic characterization of bacteria, PCR-based methods are novel, rapid, feasible and accurate one (Versalovic *et al*., 1991; Jabbari *et al*., 2002b).

Protein H is the major outer membrane protein in the envelope of *P. multocida* (Lugtenberg *et al*., 1984). This protein has been known as porin because it is structurally and functionally related to the superfamily of porins of Gram negative bacteria (Cheralier *et al*., 1993; Luo *et al*., 1997). The protein H (OmpH) is a major outer membrane protein that its role in immunogenicity and pathogenicity of *P. multocida* isolates was demonstrated (Vasfi Marandi and Mittal, 1997; Lugtenberg *et al*., 1984). The aim of this study was to determine the feasibility of the PCR-RFLP analysis of the *ompH* gene for discrimination and classification of avian *P. multocida* isolates.

MATRIALS AND METHODS

Bacterial isolates: All of the *P. multocida* isolates were recovered from fowl cholera outbreaks in northern provinces (Gilan and Mazandaran) of Iran and were described previously (Jabbari *et al*., 2002a). The somatic serotype of the isolates had previously been identified by agar gel diffusion according to Hedleston method (Jabbari *et al*., 2001)

DNA extraction and PCR amplification: The DNA was extracted from the isolates as described previously (Wilson *et al*., 1995). Briefly, bacterial cells were lysed by EDTA (0.5 M), SDS (0.5%) and proteinase K (20 ng/ml) followed by phenol- choloroform- isoamyl alcohol extraction. choloroform/ isoamyl alcohol (24:24:1) mixture. The DNA was precipitated by addition of sodium acetate and absolute ethanol. The DNA was then washed with 70% ethanol, dried at room temperature and resuspended in TE buffer (pH 8).

Two oligonucleotides based on the N-terminal sequence and downstream sequence of X-73 *ompH* gene were synthesized for PCR amplification. The sequence of the two primers which were described by Luo *et al*. (1999) are as follows:

5´- ACTATGAAAAAGACAATGGTAG-3´
5´- GATCCATTCCTTGCAACATATT-3´

PCR mixtures consisted of 20 ng of *P. multocida* genomic DNA, 60 pmol of primer A and primer B each, 0.1 mM dNTPs, 1 x PCR buffer, and 2.5 units of *Taq* DNA polymerase in 100 µl volume. Thirty-five cycles of amplification were consisted of 15s at 94°C, 1 min at 55°C and 1 min at 72°C. The amplifications began with a initial denaturation of target DNA at 94°C for 5 min and terminated by a final extension for 10 Min at 72°C.

RFLP analysis: The RFLP reaction was carried out by digesting the PCR-amplified products with *Eco*RI, *Hind*III and *Cfo*I restriction endonucleases in the supplied buffers according to the manufacturer s protocol (Roche, Germany). Restriction digestion products were separated on a 1.5% agarose gel in 1X TAE buffer at 75 V for 1.5 h. The gel was stained with ethidium bromide and visualized by a UV light box. DNA fingerprints of the isolates were compared for similarity by visual inspection of the band patterns. An alphabetic letter (A, B, C, etc.) was assigned to each pattern produced by restriction enzymes. A unique combination of restriction patterns of all enzymes was called an RFLP type (I, II, III, etc.).

RESULTS

Results of RFLP typing of avian *P. multocida* isolates are presented in table 1. Relation of RFLP groups and serotypes of the isolates are showed in table 2. The Polymerase chain reaction with *ompH* primers amplified 1.2 Kb fragment. In all 20 isolates, digestion with the restriction endonuclease *Eco*RI detected one site within the fragment resulting in two fragments of 500 and 700 bp (Fig. 1; gel A, pattern A). However five isolates namely; PMI045, PMI046, PMI026, PMI031

Table 1. RFLP types of the *ompH* amplified fragment from clinical isolates of *Pasteurella multocida* obtained after digestion with different enzymes.

RFLP Type	*EcoRI* pattern	*HindIII* pattern	*CfoI* pattern	Isolates
I	A	C	E	PMI030-PMI033-PMI034-PMI036-PMI037-PMI038-PMI039-PMI040-PMI041-PMI042-PMI043-PMI044-PMI020-PMI023-PMI024
II	A	D	E	PMI032-PMI035
III	A	D	F	PMI022-PMI047
IV	B	D	G	PMI045-PMI046
V	A	D	G	PMI025
VI	B	D	F	PMI026-PMI031
VII	B	D	E	PMI028

Table 2. Relation between RFLP groups of 25 clinical isolates of *P. multocida* recovered in North of Iran and their serotypes.

RFLP type	Serotype 1	Serotype 2	Serotype 3	Sertype 3X4	Serotype 4	Total
I	14	1	-	-	-	15
II	-	-	1	1	-	2
III	-	-	1	-	1	2
IV	1	-	1	-	-	2
V	1	-	-	-	-	1
VI	-	-	-	2	-	2
VII	-	1	-	-	-	1

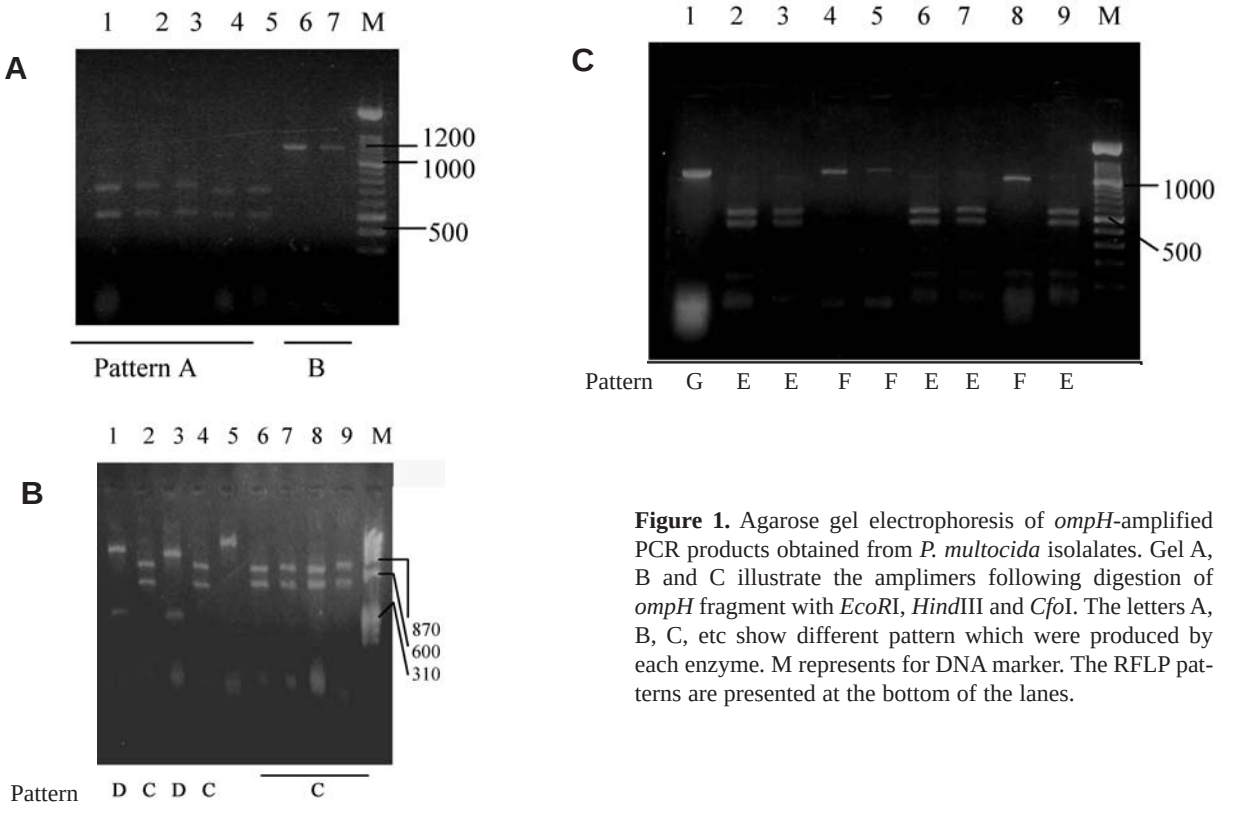

Figure 1. Agarose gel electrophoresis of *ompH*-amplified PCR products obtained from *P. multocida* isolalates. Gel A, B and C illustrate the amplimers following digestion of *ompH* fragment with *Eco*RI, *Hind*III and *Cfo*I. The letters A, B, C, etc show different pattern which were produced by each enzyme. M represents for DNA marker. The RFLP patterns are presented at the bottom of the lanes.

and PMI028 possessed no restriction site for *Eco*RI (Fig. 1; gel A pattern B). Isolates PMI026, PMI028 and PMI046 have been recovered from chicken in Mazandaran province. The origin of isolates PMI031 and PMI045 was duck from Gilan and Mazandaran provinces, respectively.

Analysis of *ompH*- amplified PCR product showed that all of the isolates examined possessed one restriction site for endonuclease *Hind*III. Digestion of *ompH* gene with this enzyme divided the isolates in two groups. Fifteen of 25 isolates showed a restriction site producing two fragments of 800 and 400 bp (pattern C). In the other group (10 isolates), *Hind*III produced two fragments of 1000 and 200 bp (pattern D). Digestion of the *ompH* fragment with restriction endonuclease *Cfo*I made *P. multocida* isolates to be separated into three groups on the basis of the presence of non, one and two sites and relative positions. Eighteen of 25 isolates possessed two sites of *Cfo*I restriction resulting in three fragments of 150, 450 and 600 bp (pattern E). Digestion of *ompH* gene in four isolates produced two fragments of 1050 and 150 bp length (pattern F). However there were three isolates, that possessed no restriction sites for this enzyme (pattern G).

Combination of different restriction patterns of *Eco*RI, *Hind*III and *Cfo*I divided 25 isolated of *P. multocida* into 7 RFLP types. Fifteen of the isolates (60%) belonged to the RFLP type I.

DISCUSSION

Recently, molecular methods which are based on genomic characteristics such as restriction enzyme analysis of DNA (Blackall *et al*., 1995; Diallo *et al*., 1995), ribotyping (Zhao *et al.*, 1992; Snipes *et al*., 1990), RAPD-PCR (Chaslus-Dancla *et al*., 1996; Hopkins *et al*., 1998) and REP-PCR (Townsend *et al*., 1997; Jabbari *et al*., 2002a) have been used for classification and discrimination of *P. multocida* isolates. Restriction fragment length polymorphism (RFLP) of a specific gene is used for genotyping of other bacteria (Shortridge *et al*., 1997; Guibourdenche *et al*., 1997; Sethi *et al*., 1997).

The immunogenicity and virulence activities of protein H has been demonstrated previously. Lubke *et al*. (1994) showed that monoclonal antibodies produced against protein H inhibited binding of *P. multocida* organisms to respiratory system. A monoclonal antibody against OmpH could passively protect mice against homologous challenge (Vasfi Marandi and Mittal, 1997). Role of OmpH as a protective antigen has been identified against homologous infection in

chicken (Luo *et al*., 1997). Vasfi Marandi and Mittal (1997), classified *P. multocida* isolates according to the OmpH epitopes by a monoclonal antibody. They reported three protein types (AI, AII, AIII) among the capsular type A isolates. As it is shown in table 2, a genetic heterogeneity was found among strains of the same serotype, for example isolates with serotype 1 placed into 3 RFLP type.

In the present study PCR analysis based on restriction sites of *ompH* gene was used successfully for genetic classification of *P. multocida* isolates. The RFLP patterns of this gene exhibited extensive restriction site heterogeneity, which may be particularly suitable for fingerprinting of *P. multocida* isolates. The patterns produced by this method had much simpler profile than those produced by genomic DNA fingerprinting (Jabbari *et al*, 2002b) allowing easy visual analysis.

In previous studies, a high degree of heterogeneity among avian *P. multocida* isolates was demonstrated by repetitive extragenic palindrome polymerase chain reaction (REP-PCR) (Guanawardana *et al.*, 2000; Jabbari *et al.*, 2002a). In this studing, we classified 25 avian *P. multocida* into 7 RFLP-PCR groups based on the pattern of *Omp*H restriction site. It was found that the vaccinal strain (PMI030) was belongeing to the RFLP type I. There were 10 isolates that belonged to six heterologous RFLP types differeing from the vaccinal strain.

Although there was a considerable genetic diversity among the strains examined, the exact relationship between RFLP type and phenotype characters such as pathogenecity and immunogenecity remain to be determined. However according to clinical history, all the strains in RFLP types I, II and IV have been isolated from acute fowl cholera outbreaks, whereas strains in RFLP-types III and VII were originated from chronic fowl cholera cases. The significance of these RFLP-PCR types in variation of virulence and other phenotypic characters among the *P. multocida* isolates remained to be elucidated.

Acknowledgment

The authors would like to thank Dr Ashtiany for his assistance and support. This research was supported by Deputy of Research and Development, RVSRI, I.R. Iran.

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