International Journal of
Dairy Science

ISSN 1811-9743

Academic
Journals Inc.

www.academicjournals.com

International Journal of Dairy Science 2 (2): 178-182, 2007
ISSN 1811-9743

Nucleic Acid Based Differentiation of *Pasteurella multocida* Serotypes

[1]P.X. Antony, [2]G.K. Nair, [2]V. Jayaprakasan, [2]M. Mini and [2]T.V. Aravindakshan
[1]Rajiv Gandhi College of Veterinary and Animal Sciences, Pondicherry, India
[2]College of Veterinary and Animal Sciences, Mannuthy, Kerala, India

Abstract: Knowledge of the serotype of *Pasteurella multocida* (*P. multocida*) involved in an outbreak is essential in order to constitute effective control measures. Antigenic characterization of *P. multocida* is accomplished by capsular serogrouping and somatic serotyping. These typing techniques are carried out by reference laboratories only. This often leads to delay in knowing the serotype of the isolate. DNA based techniques for differentiation of serotypes can provide an alternative to conventional serotyping. A PCR-REA technique was standardized to distinguish *P. multocida* serotypes A:1, A:3 and B:2

Key words: *Pasteurella multocida*, *OmpH* gene, serotypes, restriction enzyme analysis

INTRODUCTION

Pasteurella multocida has been recognized as an important Veterinary pathogen for over a century. The organism can occur as a commensal in the naso-pharyngeal region of apparently healthy animals and it can be either a primary or secondary pathogen in the disease processes of a variety of domestic and feral mammals and birds.

Conventional methods for diagnosis of pasteurellosis rely on the detection of the organism by microscopy and its isolation and identification. However as far as pasteurellosis is concerned it is not just sufficient to know the identity of the organism. To constitute effective control measures it is important to know the serotype of the organism. Typing of *P. multocida* is principally accomplished via passive haemagglutination and gel diffusion precipitin tests. Isolates are classified into five groups according to their capsular antigens and into 16 serotypes on the basis of their lipopolysaccharide antigens (Huber *et al*., 2002).

Serotyping of *P. multocida* is currently only undertaken by regional reference laboratories. A limitation of the capsule typing is the difficulty in inducing antibodies to specific antigens. Most workers find it relatively easy to make antibodies against B and E serogroup specific antigens, but not the other serogroup specific antigens. Many times a non-encapsulated strain has not been serotypeable (Rimler and Rhoades, 1989).

Recently a multiplex Polymerase Chain Reaction was introduced as a rapid alternative to capsular serotyping system (Townsend *et al*., 2001). However by this technique only the capsular serotyping information could be ascertained. Gautam *et al*. (2004) introduced a PCR technique specific for *P. multocida* serogroup A.

The PCR-Restriction fragment length polymorphism (PCR-RFLP) has been used by several workers to detect polymorphism within a gene segment and such information has been useful for serotyping of isolates.

Heinemann *et al*. (2000) investigated the use of PCR-RFLP for differentiation of serovars using 19 different restriction endonucleases and concluded that PCR-RFLP could be used as a rapid detection technique for differentiation of *Leptospira* sp. serovars.

Corresponding Author: P.X. Antony, Rajiv Gandhi College of Veterinary and Animal Sciences, Pondicherry, India
Tel: 919443956897

Restriction endonuclease analysis with *Hpa* II of 218 isolates of *P. multocida* from swine revealed 17 patterns. The authors opined that REA was a useful epidemiological tool for identifying different strains of *P. multocida* (Rubies *et al*., 2002).

Hirose *et al.* (2003) while studying the antibiotic resistance patterns of *Salmonella enterica* serovar *typhi* and serovar *Paratyphi* to fluoroquinolones demonstrated the emergence of strains with mutations in the *gyrA* genes, using PCR-RFLP.

Hong *et al.* (2003) used a PCR-RFLP flagellar typing scheme to successfully serotype 112 *Salmonella* isolates obtained from poultry. Restriction enzymes *Sau*3A and *Hha* I were used for the purpose. They concluded that PCR-RFLP was a rapid, accurate and economical approach for serotyping *Salmonella* species.

Until now no PCR-RFLP based techniques have been used for determination of both the capsular and somatic serotyping of *P. multocida*. Moreover the problems encountered in *P. multocida* serotyping system does not affect DNA fingerprinting. Hence the present study was undertaken to develop a simple DNA based typing scheme.

MATERIALS AND METHODS

Pasteurella multocida Strains

Pasteurella multocida strain DP1 and FP1 isolated in Kerala, India from a duck and fowl respectively and serotyped as A:1 by Indian Veterinary Research Institute, Izatnagar, India and *Pasteurella multocida* serotypes A:3, B:2 and a duck isolate LKO, serotype A:1 obtained from Indian Veterinary Research Institute, Izatnagar, India, formed the reference strains for this study.

Oligonucleotide Primers

Two oligonucleotides based on the sequence of *P. multocida* X-73 *OmpH* gene, Accession No. U50907 (Luo *et al*., 1997) were designed using Primer3 software. The primers were custom synthesized by M/s Bangalore Genei India. The sequences of the two primers were as follows:

OmpH 1 5'-GCG TTT CAT TCA AAG CAT CTC-3' - 21 mer and
OmpH 2 5'-ATG ACC GCG TAA CGA CTT TC -3'- 20 mer

Amplification of *OmpH* gene

PCR Conditions

A 50 μL reaction mixture was prepared in 0.2 mL thin walled PCR tube. Five microlitres of template DNA was added to a reaction mixture containing 40 pmoles each of primer *OmpH* 1 and *OmpH* 2, 200 μM of each dNTP, 1 X Taq buffer with 1.5 M $MgCl_2$ and 2 units of Taq DNA polymerase. The amplification reaction was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany) according to the following program, an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 56°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. Bacterial culture lysates was used as template DNA. The product was analysed by 2% agarose gel electrophoresis. Standard molecular size marker low range DNA ruler with fragments 3000, 2500, 2000, 1500, 1000, 600, 300 and 100 bp was used as DNA molecular size marker to ascertain the size of the amplified PCR product.

Restriction Enzyme Analysis of *OmpH*-PCR Product

The amplified PCR products were subjected to restriction enzyme digestion using restriction enzymes *Dra* I and *Hinf* I. The digestion was carried out by a standard method (Sambrook *et al*., 1989). Briefly restriction enzyme digestion was carried out at 37°C for two h, followed by inactivation of the enzyme at 80°C for 20 min. Electrophoresis of the resultant digest was conducted on 8% acrylamide gels. The gel was viewed on a transilluminator and photographed.

RESULTS

The primer pairs *OmpH* 1 and *OmpH* 2, designed to amplify the *OmpH* gene of *P. multocida* successfully amplified the *OmpH* gene of all three serotypes viz., A:1, A:3 and B:2. The amplified product had a size of approximately 1000 bp (Fig. 1).

Specificity of the Primers

Primer pairs *OmpH* 1 and *OmpH* 2 did not amplify the DNA prepared from unrelated bacterial species such as *Streptococcus zooepidemicus*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Leptospira* serogroup *canicola* serovar *canicola*.

Restriction Enzyme Analysis

Restriction analysis of the amplified products of serotypes A:1, A:3 and B:2 were carried out using the same restriction endonucleases *Dra* I and *Hinf* I. Enzyme *Hinf* I generated patterns similar in A:3 and B:2 but distinct from A:1, while *Dra* I profiles were distinct from each other for the three serotypes (Fig. 2).

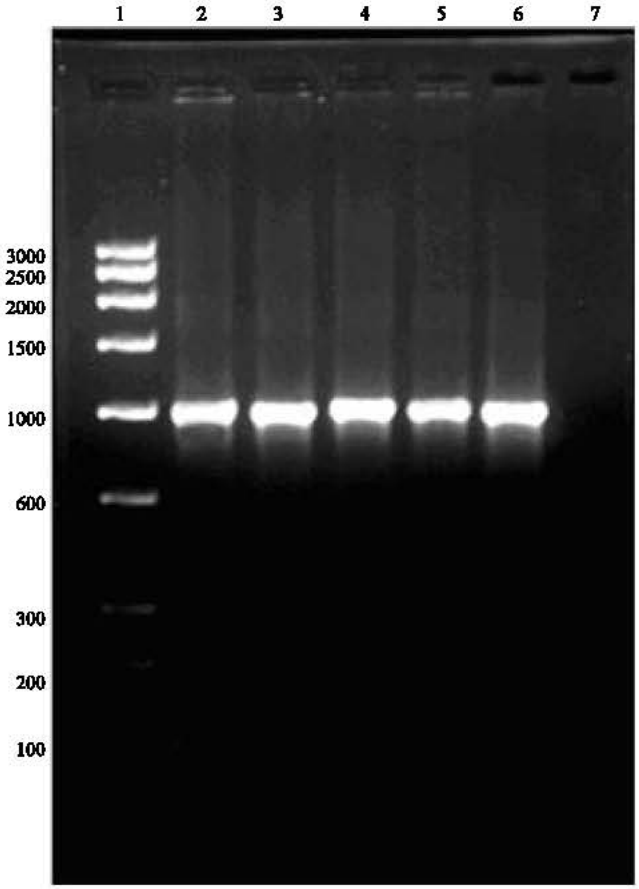

Fig. 1: Amplification of *OmpH* gene of *P. multocida* Lane 1 Low ragne DNA marker, Lane 2-3 LKO and FPI, Lane 4 DPI, Lane 5 A:3, Lane 6 B:2, Lane 7 Negative control

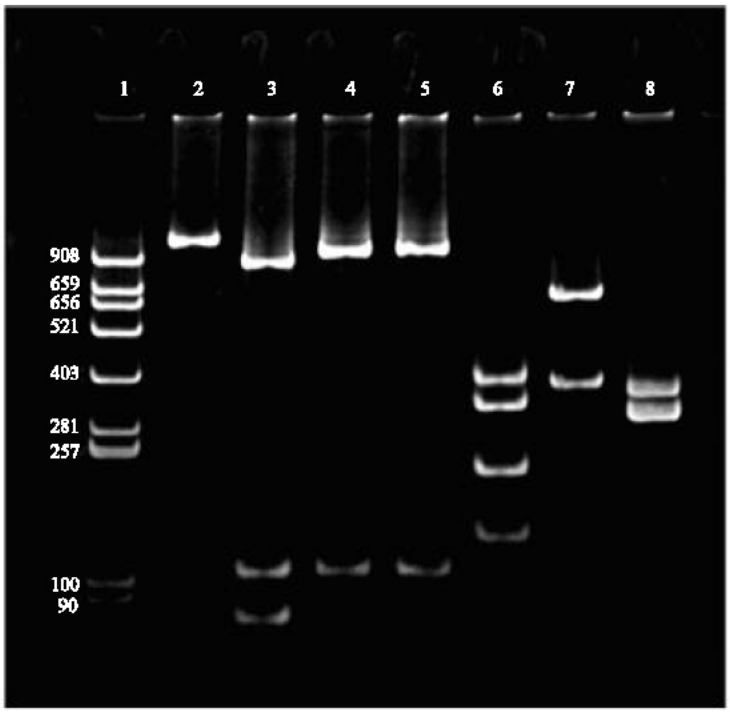

Fig. 2: Restriction enzyme profiles of OmpH-PCR products (serotypes A:1, A:3 and B:2) with *Hinf*I and *Dra* I, Lane 1 pBR 322 DNA/Alu digest marker, Lane 2 Undigested OmpH-PCR product, Lane 3-5 A:1, A;3 and B:2 (*Hinf*I digest), Lane 6-8 A:1, A;3 and B:2 (*Dra* I digest)

DISCUSSION

The custom designed primers could successfully amplify the *OmpH* gene of three serotypes viz., A:1, A:3 and B:2. Similar results were reported by Luo *et al*. (1999) who had reported successful amplification of *OmpH* genes of the serotypes 1, 3 and 4 to 16 of *P. multocida* and the product had an approximate size of 1 kilo-base pair.

REA of amplified products of OmpH-PCR with *Dra* I generated distinct profiles for the three serotypes A:1, A:3 and B:2 while Enzyme *Hinf*I generated patterns similar in A:3 and B:2 but distinct from A:1. Thus, REA of amplified products of OmpH-PCR with *Dra* I offers a novel technique for differentiation of various serotypes of *P. multocida*. If unique patterns for all serotypes could be determined in a similar manner then we can have a serotyping technique that is simple, rapid and straight forward and can be performed in any laboratory that has the capacity to perform PCR. The development of a DNA-based technique for differentiation of serotypes could provide an alternative to conventional serotyping systems and has a potential to overcome the problems associated with the current serotyping techniques, which rely on inconsistent expression of phenotypic traits.

However, further studies have to be carried out with all the different serotypes to know whether profiles unique to each serotype are obtained, before the technique can be put for routine use. To the best of our knowledge this is the first report of the use of PCR-RFLP for differentiation of *P. multocida* serotypes.

ACKNOWLEDGMENTS

The authors are grateful to the Indian Council of Agricultural Research, New Delhi, India for providing financial support under the All India Network Programme on Haemorrhagic Septicaemia and the Dean, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India for providing facilities to conduct this study. The first author also expresses his sincere thanks to the Dean. Rajiv Gandhi college of Veterinary and Animal Sciences, Pondicherry, for his keen interest and valuable suggestion during the course of this study.

REFERENCES

Gautam, K., A.A. Kumar, V.P. Singh, V.P. Singh, T.K. Dutta and S.B. Shivachandra, 2004. Specific identification of *Pasteurella multocida* serogroup A isolates by PCR assay. Res. Vet. Sci., 76: 179-185.

Heinemann, M.B., J.F. Gracia, C.M. Nunes, F. Gregori, Z.M.M. Higa, S.A. Vasconcellos and L.J. Richtzenhain, 2000. Detection and differentiation of *Leptospira* sp. serovars in bovine serum by polymerase chain reaction and restriction fragment length polymorphism. Vet. Microbiol., 73: 261-267.

Hirose, K., K. Tamura and H. Watanabe, 2003. Screening method for *Salmonella enteria* serovar Typhi and Serovar Paratyphi a with reduced susceptibility to fluroquinolones by PCR restriction fragment length polymorphism. Microbiol. Immunol., 47: 161-165.

Hong, Y., T. Liu, C. Hofacre, M. Maier, D.G. White, S. Ayers, L. Wang and J.J. Maurer, 2003. A restriction fragment length polymorphism-based polymerse chain reaction as an alternative to serotyping for identifying Salmonella serotypes. Avian Dis., 47: 387-395.

Huber, B.S., D.A. Allred, J.C. Carmen, D.D. Frame, D.G. Whiting, J.R. Cryan, T.R. Olson, P.J. Jackson, K. Hill, M.T. Laker and R.A. Robinson, 2002. Random amplified polymorphic DNA and Amplified fragment length polymorphism analyses of *P. multocida* isolates from fatal fowl cholera infections. J. Clin. Microbiol., 40: 2163-2168.

Luo, Y., J.R. Glisson, M.W. Jackwood, R.E. Hancock, M. Bains, I.N. Cheng and C. Wang, 1997. Cloning and characterization of the major outer membrane protein gene (*OmpH*) of *Pasteurella multocida* X-73. J. Bacteriol., 179: 7856-7864.

Luo, Y., Q. Zeng, J.R. Glisson, M.W. Jackwood, N. Cheng and C. Wang, 1999. Sequence analysis of *Pasteurella multocida* major outer membrane protein (*OmpH*) and application of synthetic peptides in vaccination of chickens against homologous strain challenge. Vaccine, 17: 821-831.

Rimler, R.B. and R. Rhoades, 1989. Fowl Cholera. *Pasteurella* and *Pasteurellosis*. Adlam, C. and J.M. Rutter (Eds.). Academic Press, London, pp: 95-113.

Rubies, X., J. Casal and C. Pijoan, 2002. Plasmid and restriction endonuclease patterns in *Pasteurella multocida* isolated from a swine pyramid. Vet. Microbiol., 84: 69-78.

Sambrook, J., E.T. Fritsch and T. Maniatis, 1989. Molecular Cloning. A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory, New York, pp: 5.28.

Townsend, K.M., J.D. Boyce, J.Y. Chung, A.J. Frost and B. Adler, 2001. Genetic organization of *Pasteurella multocida* cap loci and development of a multiplex capsular PCR typing system. J. Clin. Microbiol., 39: 924-929.