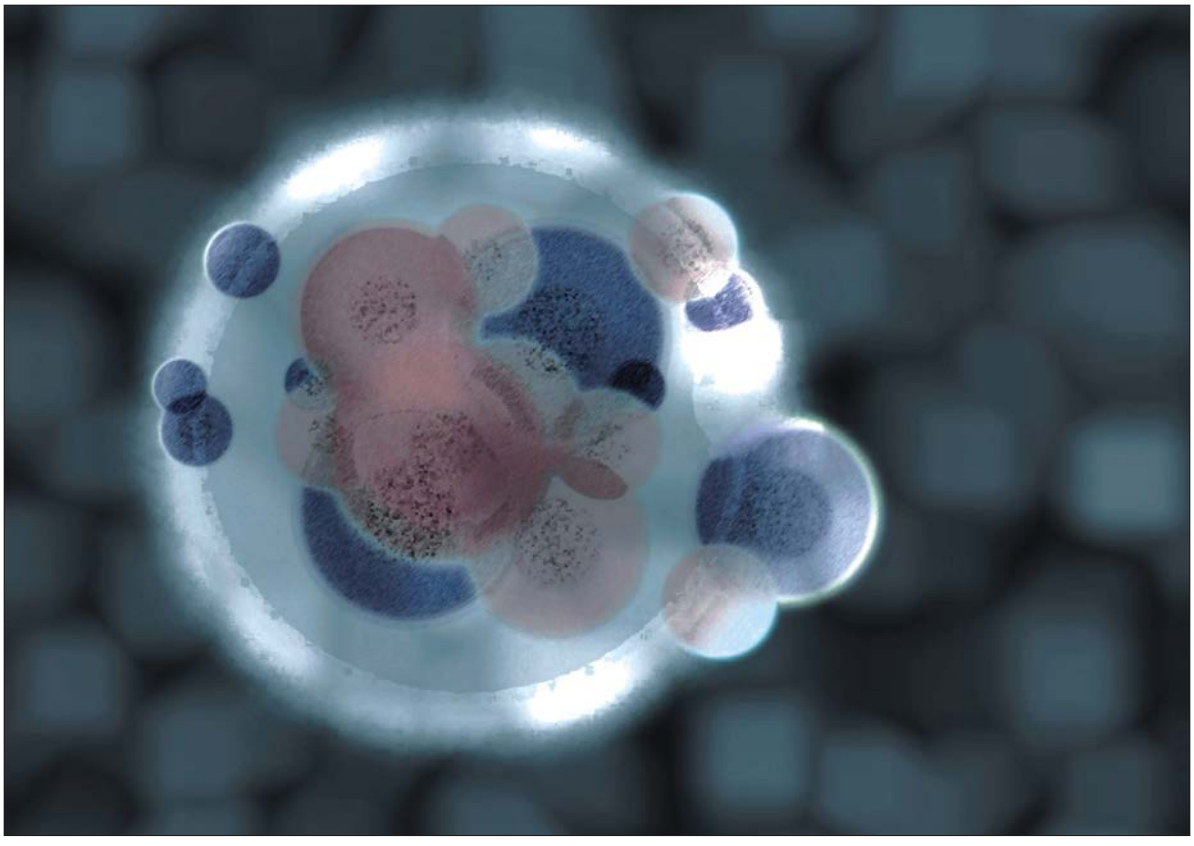

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 8 (7): 1251-1254, 2008
ISSN 1727-3048

Isolation and Identification of *Mannheimia haemolytica* and *Pasteurella multocida* in Sheep and Goats using Biochemical Tests and Random Amplified Polymorphic DNA (RAPD) Analysis

[1]A.D. Hawari, [2]D.S. Hassawi and [2]M. Sweiss
[1]Department of Nutrition and Food Processing,
[2]Department of Biotechnology, Faculty of Agricultural Technology,
Al-Balqa Applied University, Al-Salt 19117, Jordan

Abstract: The aims of this study were to isolate the *M. haemolytica* and *P. multocida* from sheep and goats of Jordan and to identify these bacteria by using biochemical tests and molecular technique. One hundred and ninety six samples were collected from nasal and throat of sheep and goats of two regions (Middle and Northern) in Jordan. The results of the biochemical tests and API 20 E kits identified 50 isolates of *Pasteurella multocida* and 5 isolates of *Mannheimia haemolytica*. The identification of these isolates was confirmed by polymerase chain reaction (PCR) technique using PMOut primers for *P. multocida* and PHSSA primers for *M. haemolytica* and by random amplified polymorphic DNA (RAPD) using the random primer (OPA-11). Fourteen RAPD profiles were found in *P. multocida* and 2 profiles were found in *M. haemolyitca*. The results of this study indicated that identification of *P. multocida* and *M. haemolytica* by RAPD analysis was in accordance with those of biochemical tests and using of more than one RAPD primers could improve the identification of the isolates.

Key words: *Pasteurella multocida*, *Mannheimia haemolytica*, RAPD analysis, PCR technique

INTRODUCTION

Pasteurellosis broadly refers to any of the disease conditions caused by species of the genus *Pasteurella* (Dziva and Mohan, 2000; Davies *et al*., 2003). *Pneumonic pasteurellosis* is an acute infectious disease that causes widespread financial losses because of death, reduced live weight, delayed marketing, treatment costs and unthriftness among survivors (Aielo *et al*., 1998; Davies *et al*., 2001; Ozbey *et al*., 2004).

Mannheimia haemolytica, formerly known as *Pasteurella haemolytica*, (Tefera and Smola, 2002; Christensen *et al*., 2003; Sisay and Zerihun, 2003) is the bacterium most frequently isolated from shipping fever, which affects sheep and goats of all ages world wide (Falade, 2002; Ozbey *et al*., 2004). *Mannheimia* species naturally inhabit the upper respiratory system (tonsils and nasopharynx) of healthy sheep and goats and other wild and domestic animals (Chen *et al*., 2002).

Mannheimia haemolytica is the etiological agent of both bovine and ovine *Pneumonic pasteurellosis* (Dziva and Mohan, 2000; Davies *et al*., 2001; Falade, 2002), although various serotypes of *Pasteurella multocida* are occasionally involved. They are non motile, non spore forming, aerobic, fermentative, gram negative coccobacilli (Kodjo *et al*., 1999; Chen *et al*., 2002) which may show bipolar staining and may grow on most laboratory media with the exception of bile containing media such as MacConkey agar (Lariviere, 1992).

Infections result when an animal is compromised by any of a variety of stress factors as inclement weather, shipping (transportation), malnutrition, bacterial invasion of host defense, viral infections, nasopharyngeal colonization and dehydration, (Radostits *et al*., 1994; Baron, 1996; Aielo *et al*., 1998). Various *M. haemolytica* virulence factors influence the outcome of bacterial-host interactions (Davies *et al*., 1997).

Bacterial species included within the genera *pasteurella* and *mannheimia* have been classified on the basis of their phytogenetic characteristics (Tefera and Smola, 2002). Capsular serotyping provides the primary basis for the classification of strains and epidemiological typing of *M. haemolytica* (Peterson *et al*., 2001). Furthermore the purified organism is subsequently classified according to phenotypic traits such as morphology, carbohydrates fermentation patterns and serological properties. However, culture conditions can influence the expression of these attributes thus

Corresponding Author: Azmi D. Hawari, Department of Nutrition and Food Processing, Faculty of Agricultural Technology, Al-Balqa Applied University, Al-Salt 19117, Jordan

diminishing the stability and reliability for phenotypic methods for strain identification (Matsumoto and Strain, 1993). So, genotyping techniques have been used extensively to differentiate epidemiologically significant strains of *P. multocida* (Lainson *et al*., 2002).

Random amplified polymorphic DNA (RAPD) analyses has been applied for the distinction of strains belonging to the same species (Williams *et al*., 1990). It is a fast, sensitive method for the epidemiological studies and PCR-based method of genetic typing based on genomic polymorphisms. Ozbey *et al.* (2004) reported that some strains of *Pasteurella multocida* and *Mannheimia haemolytica* which isolated from cattle, sheep and goats were typed by using RAPD assay.

The aims of this study were to isolate the bacteria *M. haemolytica* and *P. multocida* from sheep and goats grown in different areas of Jordan and to confirm the identification of these bacteria by using biochemical reactions and Random Amplified Polymorphic DNA (RAPD) technique.

MATERIALS AND METHODS

Samples collection: Nasal and throat swabs (196 samples) from sheep and goats were collected randomly from different locations in Jordan during 2006-2007; pneumonic lungs and tonsils from slaughter house were also collected. The swabs were placed in sterile tests tubes containing 2 mL of transport medium (Amies medium) and kept immediately in an ice box for further analysis. The samples were then cultured overnight at 37°C in Erlenmeyer flasks containing 200 mL of brain heart infusion broth.

Bacterial isolation and identification: Following incubation, samples from each culture were plated on blood agar selective medium for *P. multocida* and *M. haemolytic* (Oxoid) and on MacConkey agar. The inoculated plates were incubated aerobically and anaerobically at 37°C for 24-72 h. Following purification, through subculturing, the isolates were subjected to further identification. For the laboratory detection of *P. multocida* and *M. haemolytica*, the identification of suspected bacteria colonies was achieved by observation of colonial morphology under microscopy and the use of some biochemical tests: hemolysis, motility, indole formation, litmus milk, glucose, saccharose, lactose, oxidase and catalase. Assay for biochemical properties of the bacteria isolates were conducted according to MacFaddin's method (MacFaddin, 2000). For reliable identification and comparison of results, the API 20 E system (Biomereiux, France) was used. The bacteria were stored at -70°C in 50% glycerol in brain heart infusion broth for further use.

DNA Extraction and Polymerase Chain Reaction (PCR): DNA extraction was carried out as previously described by Ozbey *et al.* (2004) with minor modifications. Identification of *P. multocida* and *M. haemolytica* were confirmed by PCR using PMOut primers (5'- AGG TGA AAG AGG TTA TG-3' and 5'- TAC CTA ACT CAA CCA AC-3') for *P. multocida* and PHSSA primers (5'-TTC ACA TCT TCA TCC TC-3' and 5'- TTT TCA TCC TCT TCG TC-3') for *M. haemolytica* derived from Omp87 and ssa gene, respectively (Ozbey *et al*., 2004). The PCR reaction was preformed in a total volume 25 μL containing 1X Go *Taq* reaction buffer, 2.5 mM $MgCl_2$, 0.4 μM from each primer, 0.2 mM dNTPs (Bio Basic Inc., Canada), 1.25 U from Go *Taq* DNA polymerase (Promega Corporation, Madison, WI., USA) 1 μL from the isolated DNA.

The DNA was amplified under the following conditions in a PTC200 type thermocycler (MJ Research Inc., USA): denaturation step at 94°C for 2 min, 40 cycles using the following settings: denaturation at 94°C for 45 sec, annealing at 45°C for 45 sec and extension at 72°C for 1 min, followed by 5 min at 72°C. The PCR product was analyzed on 1.5% agarose gel stained with 0.5 μg mL^{-1} ethidium bromide. Two DNA ladders 1 kb and 100 bp (Bio Basic Inc., Canada) were used to determine the size of the amplified fragments.

Randomly amplified DNA polymorphism: RAPD reaction was preformed as previously described by Ozbey *et al.* (2004) in a PTC200 type thermocycler (MJ Research Inc., USA). A random OPA-11 primer (5'-CA AT CG CC GT-3') (Alpha DNA, Quebec) was used to determine the genetic differences among *P. multocida* and *M. haemolytica* isolates. Five microliter of amplified PCR products were analyzed on 1.5% agarose gel that stained with 0.5 μg mL^{-1} ethidium bromide and visualized under the UV light transilluminator. DNA molecular weight markers 1 kb and 100 bp DNA ladders (Bio Basic Inc, Canada) were used to determine the size of the amplified fragments.

RESULTS AND DISCUSSION

Gram stained of the respiratory and pneumonic lungs swabs yielded gram-negative coccobacilli. Direct leishmann's staining of respiratory and pneumonic lungs smears revealed the presence of bipolarity, which is characteristic of *Pasteurella* sp. The results of the biochemical tests (Table 1), which conducted on suspected pure colonies, identified *P. multocida* and *M. haemolytica*. These results were confirmed by using API 20 E kit; all isolates presumed to belong to *P. multocida* were produced catalase, indol, presence of ornithine decarboxylase that fermented-mannitol, acid

Table 1: Results of biochemical reactions characteristics for *M. haemolytica* and *P. multocida*

Reaction	*M. haemolytica*	*P. multocida*
Haemolysis	+	-
Motility	-	-
Indole formation	-	+
Litmus milk	Acid	Neutral
Glucose	+	+
Saccharose	+	+
Lactose	+	-
Oxidase	+	+
Catalase	+	+

+: Present, -: Not present

Table 2: Samples, isolates number and the site of isolation of *M. haemolytica* and *P. multocida*

Samples No.	Isolates No.	Animal type	Site of isolation	Isolates
122	41	Sheep	Nasopharyngeal and muco-nasal swabs	*P. multocida*
23	5	Sheep	Pneumonic lungs	*P. multocida*
7	4	Sheep	Pneumonic lungs	*M. haemolytica*
40	4	Goats	Pneumonic lungs	*P. multocida*
4	1	Goats	Pneumonic lungs	*M. haemolytica*
196	55	-	-	-

by fermentation of glucose and did not grow in MacConkey agar, while all isolates presumed to belong to *M. haemolytica* did not produce indole and grew in MacConkey agar. These results are in agreement with the findings by Tefera and Smola (2002).

A total of 55 isolates were identified out of; fifty isolates were *P. multocida* and five isolates were *M. haemolytica* (Table 2). The isolates were obtained from sheep and goats grown in two regions of Jordan, middle (42 isolates) and northern (13 isolates). On the basis of these results, *P. multocida* are the most common in sheep and goats in the middle region of Jordan. The serotypes of the isolates were not determined due to lack of serotypes in our laboratories.

The identification of *P. multocida* and *M. haemolytica* was confirmed by PCR technique. Amplification of genomic DNA from the two bacteria was conducted by using PMOut primers for *P. multocida* and PHSSA primers for *M. haemolytica*, corresponding to the anticipated sizes of 219 and 325 bp, respectively (Fig. 1). All *P. multocida* and *M. haemolytica* isolates that were positive by biochemical reactions were also detected to be positive by PCR. No amplifications were produced from the negative control. These results were in agreement with Ozbey and Muz (2006), who mentioned that All *P. multocida* chicken isolates that were positive by culture were also detected to be positive by PCR using PMOut 1-2 primers.

The profiles generated by RAPD analysis using OPA-11 primer with *P. multocida* and *M. haemolytica* isolates are shown in Fig. 2. Sixteen different profiles were found in this study; 14 profiles were found in *P. multocida* and 2 profiles were found in *M. haemolyitca*. The results of the RAPD assay indicated that more genetic heterogeneity exists among *P. multocida* and little

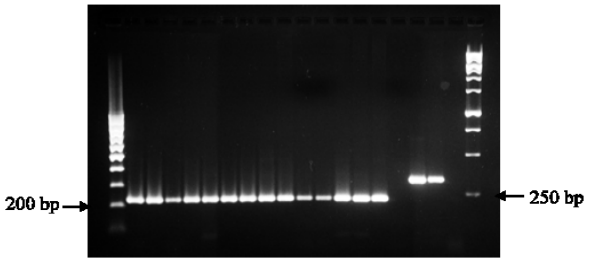

Fig. 1: PCR product for 16 samples of *Pasteurella multocida* and *Mannheimia haemolyitca* where: Lane P1-P14 are *Pasteurella multocida*, P16 and P17 are *Mannheimia haemolyitca*; P15 and P18 are negative control, M1 and M2 represent 100 bp DNA ladder and 1 kb DNA ladder, respectively

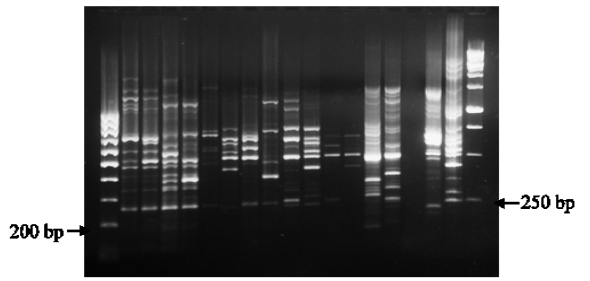

Fig. 2: RAPD patterns for 16 samples of *Pasteurella multocida* and *Mannheimia haemolyitca* where: lane P1-P14 are *Pasteurella multocida* and P15-P16 are *Mannheimia haemolyitca*; M1 and M2 represent 100 bp DNA ladder and 1 kb DNA ladder, respectively

genetic heterogeneity exists among *M. haemolytica* isolates. The results of *M. haemolytica* were in agreement with Ozbey *et al.* (2004), who indicated that little genetic heterogeneity exists among *M. haemolytica* isolates from cattle and sheep. Dziva *et al.* (2001) identified 81 *P. multocida* isolates of animal origin by both capsular typing and RAPD analysis; nine different strains with related RAPD profiles were observed. These results indicated that there was relationship between phenotypes and RAPD profiles.

The findings of this study confirmed the specificity of primers PMOut and PHSSA for *P. multocida* and *M. haemolytica*, respectively and indicated that using OPA-11 in RAPD analysis is not efficient in differentiating isolates of *M. haemolytica*. It also indicated that molecular typing methods can provide a stable and highly useful analysis of bacterial isolates and have proved to be beneficial in reducing the limitations of the biochemical tests. In conclusion, the use of more than one RAPD primers could improve the identification of isolates by RAPD technique.

ACKNOWLEDGMENTS

We thank Al-Balqa Applied University for supporting this research and we thank Jordan Bio-Industries Center for technical support.

REFERENCES

Aielo, S.E., H.E. Amstutz, D.A. Anderson, J. Sir Armour, L.B. Jeffcott, F.M. Loew and A.M. Wolf, 1998. The Merck Veterinary Manual. 8th Edn., Merck and Co. Inc., New Jersey, ISBN: 09-911910-50-6.

Baron, S., 1996. Medical Microbiology. In: Pasteurella, Yersinia, Francisella, Baron, S. (Ed.). University of Texas Medical Branch, Galveston (TX), ISBN: 0-9631172-1-1.

Chen, H.I., K. Hulten and J.E. Clarridge, 2002. Taxonomic subgroups of *Pasteurella multocida* correlate with clinical presentation J. Clin. Microbiol., 40: 3438-3441.

Christensen, H., M. Bisgard, J. Larsen and J.E. Olsen, 2003. PCR-detection of *Hemophilus paragallinarum*, *Hemophilus somnus*, *Mannheimia* (Pasteurella) *haemolytica*, *Mannheimia* spp., *Pasteurella trehalosi* and *Pasteurella multocida*. Methods Mol. Biol., 216: 257-274.

Davies, R.L., S. Arkinsaw and R.K. Selander, 1997. Evolutionary genetics of *Pasteurella haemolytica* isolates recovered from cattle and sheep. Am. Soc. Microbiol., 65: 3585-3593.

Davies, R.L., T.S. Whittam and R.K. Selander, 2001. Sequence diversity and molecular evolution of the leukotoxin (lkt A) gene in bovine and ovine strains of *Mannheimia haemolytica*. J. Bacteriol., 183: 1394-1404.

Davies, L., R. MacCorquodale, S. Baillie and B. Caffrey, 2003. Characterization and comparison of *Pasteurella multocida* strains associated with porcine pneumonia and atrophic rhinitis. J. Med. Microbiol., 52: 59-67.

Dziva, F. and K. Mohan, 2000. Pasteurellosis and *Pasteurella* in Zimbabwe: An update. Zimbabwe Vet. J., 31: 1-10.

Dziva, F., H. Christensen, J.E. Olsen and K. Mohan, 2001. Random amplification of polymorphic DNA and phenotypic typing of Zimbabwean isolates of *Pasteurella multocida*. Vet. Microbiol., 82: 361-372.

Falade, S., 2002. Further *Pasteurella* isolates from the republic of Zambia: A brief report. Trop. Vet., 20: 130-131.

Kodjo, A., L. Villard, C. Bizet, J.L. Martel, R. Sanchis, E. Borges, D. Gauthier, F. Maurinand and Y. Richard, 1999. Pulsed-field gel electrophoresis is more efficient than ribotyping and random amplified polymorphic DNA analysis in discrimination of *Pasteurella haemolytica* strains. J. Clin. Microbiol., 37: 380-385.

Lainson, F.A., K.D. Aitchison, W. Donachie and J.R. Thomson, 2002. Typing of *Pasteurella multocida* isolated from pigs with and without porcine dermatitis and nephropathy syndrome. J. Clin. Microbiol., 40: 588-593.

Lariviere, S., L. Leblanc, K.R. Mittal and G.P. Martineau, 1992. Characterization of *Pasteurella multocida* from nasal cavities of piglets from farms with or without atrophic rhinitis. J. Clin. Microbiol., 30: 1398-1401.

MacFaddin, J.F., 2000. Biochemical Tests For Identification of Medical Bacteria. 3rd Edn., Williams and Wilkins, Lippincott, New York, ISBN: 0-683-05318-3.

Matsumoto, M. and J. G. Strain, 1993. Pathogenicity of *Pasteurella multocida:* Its variable nature demonstrated by *in vivo* passages. J. Avian Dis., 37: 781-785.

Ozbey, G., A. Kilic, H.B. Ertas and A. Muz, 2004. Random amplified polymorphic DNA (RAPD) analysis of *Pasteurella multocida* and *Manheimia haemolytica* strains isolated from cattle, sheep and goats. Vet. Med. Czech, 49: 65-69.

Ozbey, G. and A. Muz, 2006. Isolation of aerobic bacteria from the lungs of chickens showing respiratory disorders and confirmation of *Pasteurella multocida* by Polymerase Chain Reaction (PCR). Veterinarski Arhiv, 76: 217-225.

Peterson, K. D., H. Christensen, M. Bisgaard and J.E. Olsen, 2001. Genetic diversity of *Pasteurella multocida* fowl cholera isolates as demonstrated by ribotyping and 16S rRNA and partial *atpD* sequence comparisons. Microbiology, 147: 2739-2748.

Radostits, O.M., D.C. Blood and C.C. Gay, 1994. Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses 8th Edn., B. Saunders Co. Ltd., London.

Sisay, T. and A. Zerihun, 2003. Diversity of *Mannheimia haemolytica* and *Pasteurella trehalosi* serotypes from apparently healthy sheep and abattoir specimens in the highlands of Wollo, North East Ethiopia. Vet. Res. Commun., 27: 3-14.

Tefera, G. and J. Smola, 2002. The utility of ENTERORapid 24 Kit for the identification of *P. multocida* and *M. haemolytica*. Vet. Med., 47: 99-103.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.