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Genotypic evaluation of *Pasteurella multocida* isolated from cattle and sheep by pulsed-field gel electrophoresis

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Abstract

Pasteurella multocida a Gram-negative bacterium exists as a commensal in the upper respiratory tracts of livestock, and poultry. It is causative agent of a range of diseases in mammals and birds including fowl cholera in poultry, atrophic rhinitis in pigs and bovine hemorrhagic septicemia in cattle and buffalo. This study aimed to isolate *P. multocida* from sheep and cattle lungs sampled and assessed by bacteriological procedures and pulse field gel electrophoresis (PFGE) characterization. In this study 52 isolated of *P. multocida* were obtained (2016-2017) from clinically healthy and diseased animals (sheep and cattle) evaluated by PFGE for determining the relationship between them. According to the results of this study 12 sheep isolates had similarities above 94.00% and two cattle isolates showed similarities above 94.00%. When compared between sheep and cattle, most isolates showed a similarity of less than 50.00% indicating the great differences between isolates. It is noteworthy that in the present study, performed by PFGE to determine the type of *P. multocida* isolates, a very high distinction was made to determine the type of isolates and the relationship between isolates based on fragments in their genome using enzymes.

Introduction

Pasteurella multocida colonizes the upper respiratory tract and gastrointestinal tract of many domestic and wild animals around the world. *P. multocida* is a Gram-negative, non-motile, penicillin-sensitive coccobacillus bacterium with positive oxidase, catalase and indoletests.[1] In clinical specimens using Leishman stain, methylene blue or Giemsa, it is in a bipolar rod state. The isolates of *P. multocida* are aerobic or facultative anaerobic and grow well in nutrient media. *Pasteurella multocida* strains are classified into different groups based on different characteristics such as pathogenicity, host type, antigen and serum properties, and biochemical and culture characteristics. The strains are divided into five serum groups based on capsule antigens (A, B, D, E and F) and 16 serotypes based on lipopolysaccharide antigens.[2-4]

Pasteurella species cause economically important endemic diseases in a wide range of domestic and wild animals and birds. *Pasteurella multocida* is one of the most common opportunistic pathogens in the upper respiratory tract in domestic and wild animals including cattle and buffaloes,[5,6] chicken,[7-9] turkey[9] and other wild birds,[10,11] pigs,[9,12] dogs[9] and cats[9,12] (including domestic cats as well as large feral cats such as tigers, leopards and lions). Bacterial transmission occurs through direct contact with nasal secretions which can be caused by chronic infection in upper respiratory tract. Concomitant or pre-existing infection with other respiratory pathogens, especially *Bordetella bronchiseptica*[13] or *Manheamia hemolytica,*[14] significantly increased the colonization of *P. multocida* in the lower respiratory tract and led to severe respiratory disease. Primary infection with respiratory viruses or mycoplasmas was also a factor that leds to secondary infection with *P. multocida* or *M. hemolytica*.[14-17]

The diagnosis is based on the isolation of bacteria from clinical specimens. Bacterial colonies are identified based on appearance and microscopic characteristics, preparation of smear and Gram-staining, and biochemical tests.[1] In recent years, the identification and classification of *Pasteurella* spp. have been based more on genotypic characteristics. Molecular methods such as DNA hybridization and nucleic acid amplification allow these bacteria to be detected directly from clinical specimens, thus, effectively reduce the detection time.[18]

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Diagnostic methods based on polymerase chain reaction (PCR), using specific primers that identify target genes, are important in diagnosing infections caused by *P. multocida*. The collection of data from genomic studies identified a unique region that, after designing a pair of primers for it, amplified a 460 bp fragment for all types of *P. multocida* serotypes tested.[19]

Typing methods based on epidemiological studies such as biotyping, pattern of outer membrane proteins and phage typing methods are time-consuming and often have vague results. In recent years, the use of genetic traits with high adaptability not only speeds up the detection of an organism, but also determines the classification position of the organism in the evolutionary path chain.[18,20]

Investigation of restriction enzymatic digestion products based on pulse field gel electrophoresis (PFGE) is a specialized method for determining the type of isolates of different organisms. In this method, the DNA of the target organism is cut with a restriction enzyme so that a limited number (5 to 20 pieces) of DNA fragments with a size of about 10 to 800 kbp are produced. Then, digested DNA is separated by the PFGE and the pattern of the band created by the specific software is evaluated and compared.[21]

Because there is a great variety between isolated strains of *P. multocida* in different geographical areas of different animals, it is necessary to study this diversity and prepare a suitable genetic model to compare strains of *P. multocida*. Therefore, this study aimed to use PFGE to determine the genetic characteristics of *P. multocida* isolated from sheep and cattle and to compare the obtained patterns.

Materials and Methods

Sample collection. From the early winter of 2016 to the spring of 2017, 120 sheep and cattle lungs were sampled in the Shiraz slaughterhouse. Samples were collected from lungs with pathological lesions (hepatization), from cranioventral lobes. Lung samples were carried to the laboratory under aseptic conditions and in sampling containers. Also, several *P. multocida* isolates stored in the microbiology archives of Shiraz Veterinary School were examined.

Cultivation and isolation of samples. In the laboratory and under aseptic conditions, the surfaces of the lung specimens were sterilized using heat and the tissue was cut with a sterile surgical blade and the sample was taken from inside the slit using a sterile swab. Then, swabs were cultured on blood agar medium (Merck, Darmstadt, Germany) containing 5.00% defibrinated sheep blood and incubated at 37.00˚C for 24 - 48 hr. For initial isolation, colonies suspected of being *P. multocida* on the blood agar medium were removed and sub-cultured. Then, smears prepared from the grown colonies were Gram-stained. Biochemical tests including catalase, oxidase, indole production, motility, H_2S production, fermentation of sorbitol, glucose, mannitol and maltose (LabMal, Selangor, Malaysia) were performed for colonies that showed proper characteristics in Gram-staining. The biochemically identified samples were tested for final confirmation using specific primers (Gene Fanavaran Co., Tehran, Iran) of *P. multocida kmtI* gene.[22]

DNA extraction. The isolated samples were cultured overnight in brain-heart infusion (BHI) (Merck) broth at 37.00˚C and then used for DNA extraction by boiling method. For this purpose, 2.00 mL of the bacterial suspension was centrifuged at 12,000 rpm for 4 min. The supernatant was then completely drained and 200 µL of sterile distilled water was added to the precipitated cells. The centrifugation was performed again as before, then, 100 µL of sterile distilled water was added to the cell pellet and the cell suspension was placed in a boiling water bath for 10 min. Then, the cell suspension was placed on ice for 5 min and the centrifuge was done for 5 min at 12,000 rpm. Finally, 50.00 µL of the supernatant was transferred to a clean microtube as a DNA source and stored at –20.00˚C.[23]

Preparation of PCR mixture. To prepare the reaction mixture with a volume of 25.00 µL, the various components were mixed as follow: 2.50 µL of 10X PCR buffer, 1 µM of a mixture of four deoxynucleotides (dNTPs), 1.00 µL of *Taq* DNA polymerase, 1.00 µL of each forward and reverse primers (CinnaGen Co., Tehran, Iran), 3.00 µL of extracted DNA and double sterile distilled water to 25.00 µL. After mixing, brief centrifugation was performed for 5 sec and the microtubes were placed in a thermocycler (Analytik GmbH, Jena, Germany). Primers used included KMT1T7 (5′ATCCGGCTATTTACCCAGTGG3′) and KMT1SP6 (5′GCTGTAAACGAACTCGCCAC3′).[22] The annealing temperature for primers was 56.00˚C the amplicon size of the gene was460 bp. *P. multocida* 85020 (wild-type serotype B: 2) and Quetta (serotype B: 2 vaccine strain) were used as control.

Electrophoresis. Agarose gel (1.50%) was used to isolate and observe the PCR product. For this purpose, 5.00 µL of PCR product was mixed with 1.00 µL of loading buffer and loaded in gel wells to which Safe stain was added. For preparing agarose gel and electrophoresis, 0.50X Tris borate with EDTA (TBE; Merck) buffer was used. Electrophoresis was performed with a voltage of 85.00 - 90.00 v for 45 min. The gel was then placed on an ultraviolet transilluminator (BTS, Tokyo, Japan) and the bands were viewed and photographed.

Pulsed-field gel electrophoresis procedure. *P. multocida* and *Salmonella Braenderup* (as the ladder for PFGE) were cultured on a trypticase soy agar (Merck) medium containing 5.00% sheep blood and incubated at 37.00 ˚C for 18 hr. Then, few bacterial colonies were suspended in the test tube containing 2.00 mL of cell

suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.00) and the optical density of suspension was adjusted between 0.80 and 1.00 at 610 nm. Then, 100 µL of the bacterial suspension was transferred to a clean microtube and 5.00 µL of proteinase K (CinnaGen) and 100 µL of 1.00% (w/v) agarose LF™ (X174; Amresco, Solon, USA), which liquefied in a hot water bath at 55.00 - 60.00˚C, were added to the microtube and gently mixed with pipetting. The suspension was transferred to the molds without creating bubbles and placed at 4.00˚C for 5 min to solidify. The prepared plaques were transferred to a 50.00 mL Falcon containing 5.00 mL of cell lysis buffer (50.00 mM Tris, 50.00 mM EDTA, pH 8.00, 1.00% sarcosyl; Takara Bio, USA) and 25.00 µL of proteinase K and placed at 55.00 - 56.00˚C in a shaker incubator at 150 rpm (*Salmonella* for 2 hr and *P. multocida* for 18 - 20 hr). Before these steps, the *P. multocida* plaque was incubated in 1.00mL of Tris EDTA buffer plus 10.00 µL of lysozyme at 37.00 ˚C for 3 - 4 hr. Cell lysis buffer was drained and 10.00 mL of clinical laboratory reagent water (CLRW) was added to Falcon and placed in a shaker incubator at 54.00˚C for 15 min at 150 rpm. This washing step with CLRW was done twice. In the next step, CLRW was drained and 10.00 mL of TE buffer was added and placed at 54.00˚C for 15 min in a shaker incubator at 150 rpm. After the last step of washing, the contents of the Falcon were emptied and a new buffer was added and the plaques were kept at 4.00˚C. For enzymatic digestion, *S. Braenderup* H9812 plaque was sliced with a sterile scalpel blade 2.00 mm wide and added to the microtube containing a mixture of 173 µL of CLRW, 20.00 µL of buffer, 2.00 µL of bovine serum albumin (BSA; Glentham Life Sciences Ltd., Corsham, UK) and 5.00 µL (50 units) of *Xba*I enzyme (Takara Bio Inc., Shiga, Japan), and incubated for 90 - 120 min at 37.00˚C. Also, 175 µL of CLRW, 20.00 µL of buffer, 2.00 µL of BSA, and 5.00 µL (50.00 units) of *Sma*I enzyme (Takara Bio Inc.) were added to *P. multocida* plaque slice, and incubated at 25.00˚C for 12 hr. Then, buffer and enzyme were replaced by 200 µL of TE buffer and samples were prepared for electrophoresis. The agarose LF™ was prepared in 0.5X TBE buffer. The agarose gel was formed in an acrylic mold. After complete solidification, the appropriate restricted agarose slices and DNA markers (*S. Braenderup* H9812 digested with *Xba*I) were loaded in the wells of the agarose LF™. The wells were then sealed with molten (56.00 ˚C) 1.00% (w/v) agarose gel. The electrophoresis tank, containing 0.5X TBE buffer, was allowed to cool to 14.00˚C. The prepared agarose gel was gently set into the electrophoresis tank and the obtained restriction fragments were separated according to their size by PFGE using a contour-clamped homogeneous electric field technique (CHEF-DRIII; BioRad Laboratories, Richmond, USA) with switch times of 12.60 - 40.10 sec, a voltage of 6.00 v cm^{-1} and an involved angle of 120′ at 14.00 ˚C for 19 hr. After electrophoresis, the agarose gel was stained in Safe stain solution (10.00 µL of Safe stain in 500 mL of sterile distilled water) with gentle shaking for 30 min. The agarose gel was visualized using the ultraviolet trans-illuminator. The gel was photographed with the Gel Doc XR system (Bio-Rad, Hercules, USA). Banding patterns were visually compared by eye and grouped according to interpretative PFGE criteria. The PFGE profiles were analyzed by Bionumerics Software, (version 7.1; Applied Maths, Sint-Martens Latem, Belgium). The Dice similarity coefficient with a UPGMA (unweighted pair group method with arithmetic mean) dendrogram was produced based on 1.00% tolerance and optimization. An 80.00% cutoff line was considered to analyze genetic relatedness.

Results

In this study, 120 samples of sheep and cattle lung tissue taken from Shiraz slaughterhouse were examined to isolate *P. multocida*. Also, *P. multocida* stored in the archives of the Bacteriology Department of Pathobiology, Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran, was used. In initial isolation and identification using culture and biochemical tests, 23 samples were isolated from 120 lung tissue.

Based on biochemical tests, all isolates were positive for oxidase and catalase and indole production. Also, these isolates were non-motile and could not produce H_2S. Based on the fermentation ability of mannitol, sucrose, glucose, lactose and maltose all isolates were identified as *P. multocida*.

Based on PCR test to identify and confirm the isolates of *P. multocida* using species-specific primers by producing 460 bp amplicon, 52 samples were confirmed [23 samples of lung tissues and 29 isolates (13 of them were bovine isolates) from the archives of the Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran.

The PGFE was performed to determine the pulsotypes of 52 *P. multocida* isolated from cattle and sheep. Fifteen isolates from cattle and 37 isolates from sheep were examined (Figs. 1A and 1B). The resulting band patterns were analyzed by visual inspection followed by analysis with BioNumerics Software for relatedness evaluation. Dendrograms were generated from similarity matrixes calculated with the Dice coefficient and patterns were clustered by the unweighted pair group method with arithmetic averages using optimization and a tolerance of 1.00%. Profiles with more than 80.00% similarity were considered closely related.

In the analysis of *P. multocida* sheep isolates with a similarity percentage above 94.00% for pulsotyping, 12 isolates showed similarity above 94.00% and 25 isolates had similarity above 80.00%. Also, in the similarity above 94.00%, there were six pulsotypes, bearing 2 sub-clusters in clusters 3 and 4. The two sheep isolates showed 98.40% similarity (Fig. 2).

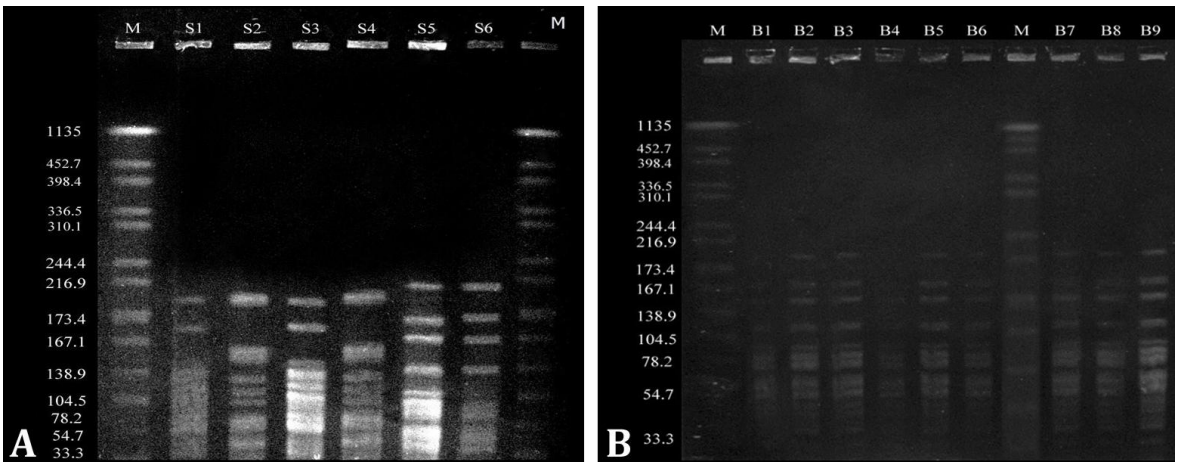

Fig. 1. A) Sample of PFGE gel digested by *Sma*I enzyme. Lanes M: Molecular size marker of PFGE (*S. Braenderup* H2812 digested by *Xba*I). Lanes S1 - S6: Some sheep isolates of *P. multocida*. **B)** Sample of PFGE gel digested by *Sma*I enzyme. Lanes M: Molecular size marker of PFGE (*S. Braenderup* H2812 digested by *Xba*I). Lanes B1 - B9: Some bovine isolates of *P. multocida*.

Comparing between cattle isolates of *P. multocida* and drawing a PFGE dendrogram, the results were as follow: Considering the similarity percentage above 94.00% for pulsotyping of isolates, only two isolates showed more than 94.00% similarity (97.90%) and with a similarity percentage greater than 80.00% five isolates had a similarity greater than 80.00% (Fig. 3). With a similarity of 94.00% and above only one cluster was identified and with a similarity of 80.00% and above it contained 2 clusters. The cluster number 2 had two sub-clusters.

Comparing *P. multocida* isolated from cattle and sheep using dendrogram and analysis of PFGE bands. It was found that there was 51.50% similarity only in 4 cattle and sheep isolates (Fig. 4). This suggested that there was a significant difference between DNA-cut patterns of bovine and ovine isolates of *P. multocida* using the *Sma*I enzyme.

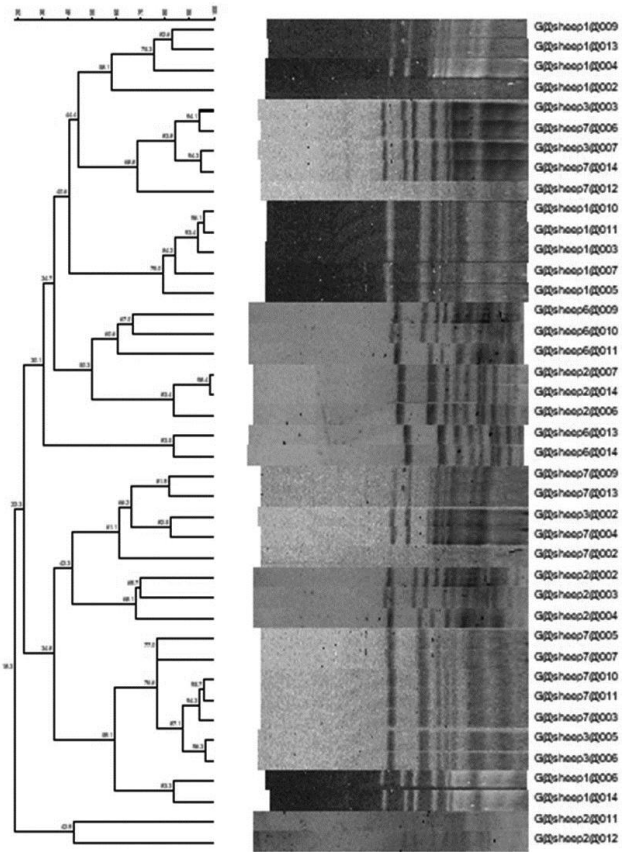

Fig. 2. PFGE dendrogram chart of *P. multocida* sheep isolates.

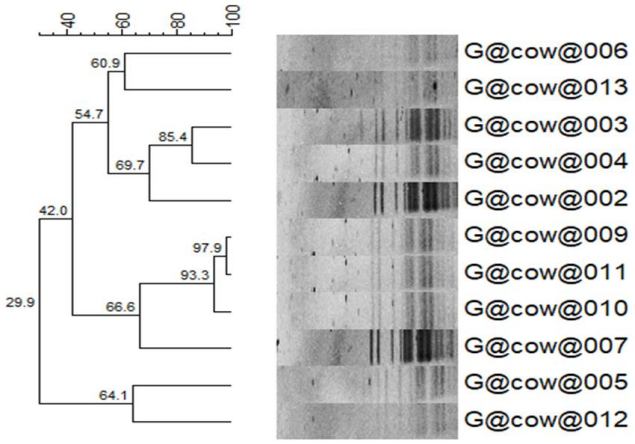

Fig. 3. PFGE dendrogram chart of *P. multocida* cattle isolates.

Discussion

Pasteurella multocida is one of the most troubling bacteria in animals. This can also be transmitted to human. It is mostly founded in the upper respiratory tract thought to be present in respiratory infections. However, other mechanisms are usually responsible for the progression of bronchiectasis and *P. multocida* may be a secondary factor. However, finding *Pasteurella* in sick animals is of particular importance to humans. This bacterium is one of the predominant pathogens and people who are exposed to infected animals are more at risk.[24]

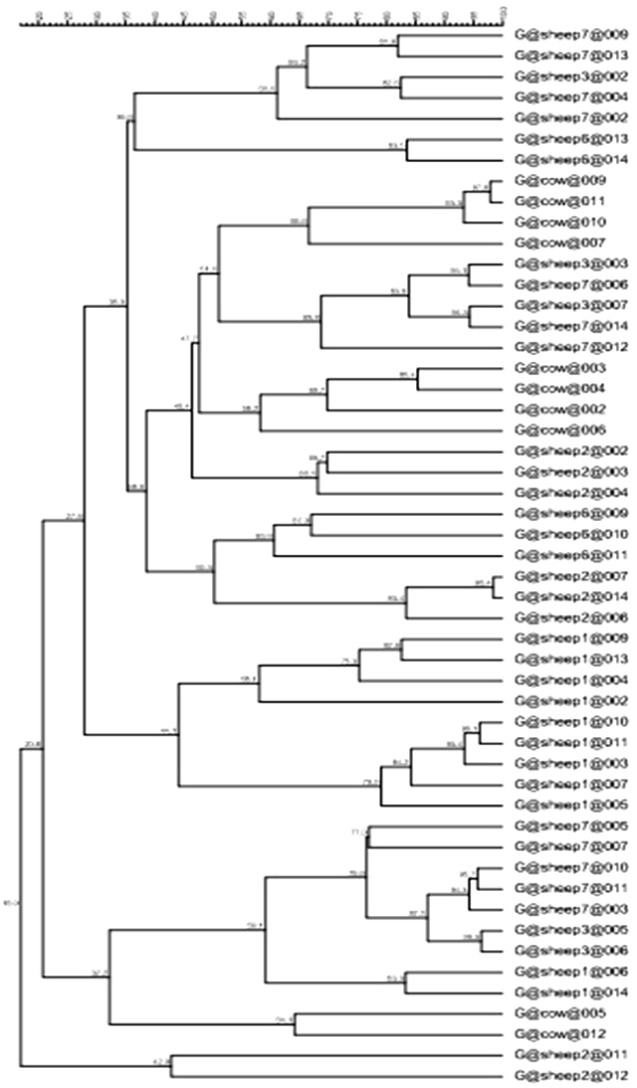

Fig. 4. PFGE dendrogram chart of cattle and ovine isolates of *P. multocida*.

In a study of 233 *P. multocida* isolated from 2,912 pigs with respiratory symptoms in China, *P. multocida* was the fourth most common pathogen in the respiratory tract of animals with pneumonia after *Streptococcus swiss, Haemophilus parasuis* and *Escherichia coli*.[25]

Jaglic *et al*. worked on the identification of *P. multocida* isolated from rabbits by pulse field gel electrophoresis. In that study, 207 isolates of *P. multocida* were examined and DNA samples were digested using *Apa*I restriction enzyme. The presence of capsules and fimbriae and hemolytic activity and enterotoxin production in the presence of rabbit plasma was also tested. Based on the results, there were twenty different patterns in which all isolates had capsules and only four isolates had fimbriae.[26]

In Thailand Worarach *et al.* investigated the genetic characteristics of *P. multocida* isolated from cattle and buffaloes using pulse field gel electrophoresis (PFGE). They worked on 87 specimens isolated during 1989 to 2011 in comparison with Thai and Laotian vaccine strains. The results of serotyping showed that the majority of the isolates (88.50%) belonged to serotype B: 2 (n = 77) while 11.50% shared with serotype B: 2, 5 (n = 10). *Apa*I digested PFGE revealed a high degree of homogeneity. Eighty-one isolates were PFGE type 4 (93.10%), as well as the Thai and Laotian vaccine strains, 4 isolates were PFGE type 1 (4.60%), 1 isolate was PFGE type 2, and 1 isolate was PFGE type 3. In conclusion, a remarkable homogeneity was observed among the HS isolates caused by *P. multocida* in Thailand over the past 22 years.[27]

Another study was done on pneumonic bats in Germany. Eighty-one *Pasteurella* spp. were isolated and identified from lung lesions. Thirty specimens were identified as *P. multocida* which showed similar patterns using PFGE and *Sma*I enzyme and the resulting data.[28]

A study by Subaaharan *et al*. on 63 isolate of *P. multocida* isolated from birds infected with fowl cholera was performed in Australia. In that study, three other isolates were considered as standard strains. The serotyping was determined with adk, est, gdh, pgi, pmi and zwf primers that detected fragments between 570 and 784 bp. Their results were such that a total of 29 different patterns were created from 66 isolates. There was also a strong correlation between the results of multilocus sequence typing (MLST) when compared to the results of ribotyping performed on isolates. In addition, the results of MLST were highly consistent with PFGE and Repetitive element sequence-based PCR (rep-PCR) indicating that MLST provided a high level of information for studying the epidemiological structure of *P. multocida*.[29]

In a study conducted in UK on pigs with atrophic rhinitis isolated *P. multocida* were compared to PFGE and MLST methods. In that study, 111 pig isolates were examined using PFGE and ApaI restriction enzyme, and 20 to 29 capsule serotypes were identified by MLST. The PFGE results showed that 15 clusters were identified that were 50.00% similar to each other and all isolates had capsule A serotype. Also, there was no genetic correlation between the identified isolates in comparison with the lesions.[30]

In another study, 95 isolates of *P. multocida* were studied using PFGE and *Apa*I restriction enzyme. Seventy-three samples from Australia and 22 samples from Vietnam were analyzed most of which were capsular type A isolates. The results showed that 21 different patterns were identified among the isolates taken from Australia, and only three patterns were created among the isolates taken from Vietnam. Also, when comparing PFGE results with rep-PCR, it was found that rep-PCR showed less information than PFGE for the classification of isolates.[31]

It is noteworthy that in the present study which was performed by PFGE to determine the type of *P. multocida* isolates, a very high distinction was made to determine the type of isolates and the relationship between isolates

based on fragments in their genome using enzymes. Specified the limited to create a fingerprint. Since this method showed the same pattern with repetitions on each isolate and had high reproducibility by examining the patterns obtained from isolates taken from different animals, there were many genotypic variations between them and a very small percentage of isolates were similar.

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Conflict of interest

There is no conflict of interest to declare.

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