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Research Article

Molecular Characterization of Isolated *Mannheimia haemolytica* and *Pasteurella multocida* from Infected Sheep and Goats Using RAPD and ERIC Markers

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Abstract

Background and Objective: *Mannheimia haemolytica* (*M. haemolytica*) and *Pasteurella multocida* (*P. multocida*) are non-motile, Gram-negative coccobacilli bacteria responsible for considerable economic losses in farm animals. This study aimed to use the random amplification of polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC) markers for characterization and differentiation between *M. haemolytica* and *P. multocida* and whether, they can be used as diagnostic tools. **Materials and Methods:** Blood and lungs samples were collected from infected sheep and goats. Biochemical tests were used to identify the isolated microorganism. The extracted DNA from the positive isolates was used for the molecular characterization using RAPD and ERIC markers. **Results:** The biochemical tests showed 11 positive isolates of *M. haemolytica* and 6 positive isolates of *P. multocida*. The molecular characterization of *M. haemolytica* and *P. multocida* showed genetic heterogeneity between the isolates. The different RAPD markers showed different molecular patterns between *M. haemolytica* and *P. multocida*. The result of ERIC marker is characteristic for each Pasteurellaceae species. The ERIC assay patterns presented obvious differentiation between the two species. **Conclusion:** The RAPD markers clarified that the molecular characterization of *M. haemolytica* and *P. multocida* was genetic heterogeneity. ERIC marker is effective molecular tool for epidemiological studies and differentiation between Pasteurellaceae family (*M. haemolytica* and *P. multocida*).

Key words: *Mannheimia haemolytica*, *Pasteurella multocida*, RAPD, ERIC, pneumonia

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Severe economic losses are resulted from the infection by *Mannheimia haemolytica* and *Pasteurella multocida* in ruminant and poultry. The disease can attack all ages of sheep and goats causing high morbidity and mortality. It is also considered as the major bacterial agent of bovine respiratory disease (BRD) complex, which is the cause of over \$3 billion in losses to the cattle industry every year¹⁻³.

Veterinary services (seromonitoring) and accurate diagnosis of microorganisms are varying widely depending on local facilities and techniques adopted. Applications of several conventional diagnostic techniques are slow, not sensitive and could not distinguish the members of microorganisms. Molecular biology tools have been qualified to support the microbiology exploration. Recently, molecular identification of the pathogen is developed and become sensitive and accurate approach for detecting microbes universally⁴⁻⁸. Accordingly, the approach to the molecular characterization of the different pathogens should be developed and widely used in the local veterinary services.

The different DNA methods reported for molecular microbiology and virology characterization are Southern hybridization analysis using specific DNA probes, microarray technology, plasmid profiling and polymerase chain reaction (PCR)-based methods⁹. PCR is the most widely used technology in the molecular biology. Its approach is developed to distinguish the specific target microbes. Random amplification of polymorphic DNA (RAPD) marker is an 8-10 nucleotide length used for amplification of random segments of genomic DNA¹⁰⁻¹². RAPD marker has the advantage to differentiate between genetically distinct individuals and it does not need to obtain sequence data for primer design, which makes experimental manipulation much easier compared with other techniques^{13,14}. Many previous studies reported a value used of RAPD markers for genotyping *P. multocida* and *M. haemolytica*¹⁵⁻¹⁸. Two previous studies were used the RAPD markers for the molecular differentiation between *P. multocida* and *M. haemolytica*^{19,20}. The two studies used one to three RAPD markers, however, researchers were able to define marker clearly distinguished between the two members of the Pasteurellaceae family.

Enterobacterial repetitive intergenic consensus (ERIC-PCR) is a genetic marker used for molecular typing the microbial microorganisms⁹. The ERIC marker depends on the highly conserved central inverted repeats of 126 bp located in extragenic regions of bacterial genomes. The position of

these elements in enterobacterial genomes varies between different species and strains^{9,21}. However, detection of the microorganism fingerprint is an important tool for characterization and differentiation of the microbial microorganisms few studies using ERIC were recorded for *P. multocida*^{21,22} while study the *M. haemolytica* using ERIC marker was not reported.

This study aimed to use the RAPD and ERIC markers to molecular characterization and differentiation between *M. haemolytica* and *P. multocida* and whether, they can be used as diagnostic tools.

MATERIALS AND METHODS

Animals: The present study was performed on small density farms of sheep and goats resident in Giza governorate of Egypt in the summer season of 2017. The farm capacities range from 5-30 animals. The animals suffered from high mortality rate, fever with respiratory manifestations including dyspnea, difficult breathing, nasal discharging, coughing as well as rales with auscultation.

Also, postmortem lung samples were collected from El-Moneeb abattoir. These samples were from the lung parts that showing hemorrhagic bronchopneumonia accompanied by pleurisy especially in anterior and ventral segments together with pericarditis.

Samples collection: The collected samples were 30 blood samples from clinically affected sheep and goats (20 sheep and 10 goats) and 20 lung tissues samples from slaughter sheep.

Bacterial isolation: Swapped of the lung tissues specimens and blood samples were inoculated into the blood agar containing 5% blood sheep, MacConkey's agar and nutrient broth and incubated at 37°C for 24 h. The obtained colonies were morphologically and biochemically identified according to James²³.

Biochemical tests: All the isolates were tested biochemically by inoculation of the peptone water grown culture of each isolate in 1% glucose, sucrose, sorbitol, manitol, fructose, dulcitol, lactose, silicin, arabinose and maltose, incubated aerobically at 37°C for 72 h. Indole, oxidase, catalase, urease production and nitrate reduction tests were carried out according to their standard bacteriological procedure²⁴.

Pathogenicity test: Seven isolates (3 isolates for *M. haemolytica* and 4 for *P. multocida*) were inoculated intra-peritoneally in 6 weeks old Swiss albino mice. A total of 3 mice were used for each isolate. The 10^{-1} mL of inoculum containing 0.3×10^8 organisms mL^{-1} was used for the mice inoculation²⁵. The control mice were inoculated with 0.1 mL of sterile saline. All the mice were observed and the mortality was recorded. Blood smears were prepared from the heart blood of dead mice and stained with Leishman's stain. Re-isolation of Pasteurellaceae species from heart blood of the dead mice was conducted on sheep blood agar²⁶ and biochemical tests.

DNA extraction: The positive isolates for *M. haemolytica* and *P. multocida* were cultured overnight in the brain-heart infusion (BHI) broth (Oxoid, United Kingdom) at 37°C. Bacterial genomic DNA was extracted according to Ozbey *et al.*¹³. The DNA concentration and purity were measured by Nano-drop.

Molecular study: Four RAPD and ERIC markers were used. Table 1 illustrates the sequences of the markers used. A PCR cocktail consisting of 1.0 μM of each primer and 2x power of PCR master mix (iNtRON Biotechnology, Korea) were placed into PCR Eppendorf tubes with 100 ng of DNA. The reactions ran in a Coy Temp Cyclor II (Biorad, USA). RAPD-PCR reaction was cycled for 5 min at 94°C, 45 cycles of 94°C: 1 min, 36°C 1 min, 72°C: 2 min, extension at 72°C for 5 min.

The enterobacterial repetitive intergenic consensus marker was used according to Ahmed *et al.*²². The PCR program was denaturation at 95°C for 10 min, then 30 cycles at 94°C for 1 min, 52°C for 1 min, extension at 65°C for 8 min and a final extension at 65°C for 16 min.

The PCR products were electrophoresed on 2% agarose gel (Invitrogen Ultrapure TMAgrose®-Carlsbad, USA) together with a 50 bp DNA ladder (Promega Corporation, France) for molecular weight estimation. The amplified products were visualized in gel documentation apparatus, photographed and analyzed.

Table 1: Primers sequences used for RAPD and ERIC assay

Code	Sequences of primer (5'-3')
OPA-4	AATCGGGCTG
OPA-9	GGGTAACGCC
OPA-20	GTTGCGATCC
OPC-5	GATGACCGCC
ERIC (F)	ATGTAAGCTCCTGGG GAT TCA
ERIC (R)	AAGTAAGTGACTGGGGTGAGCG

Determination of genetic variation: Genetic diversity within each strain for *M. haemolytica* and *P. multocida* were determined as the observed numbers of band and the molecular bands size using 50 bp DNA ladder.

RESULTS

Biochemical identification: All the isolates were identified biochemically and the results are illustrated in Table 2. The isolates of *M. haemolytica* showed Gram-negative rods, did not produce Indole, grew on MacConkey's agar and grows on the blood agar with hemolytic effect while the isolates of *P. multocida* observed Gram-negative rods, positive to Indole, did not grow on MacConkey's agar, grows on the blood agar without haemolytic effect. The biochemical identification of the isolated samples revealed that 9 out of 40 sheep's samples (20 blood samples and 20 lung tissue samples) were identified as *M. haemolytica* and 5 were *P. multocida*. While the goats' samples (10 blood samples) showed 3 *M. haemolytica* positive isolates and 1 isolate identified as *P. multocida*.

Pathogenicity results: The 8 mice groups that were injected with the seven isolates in addition to the control group were observed. Excluding the control group and the inoculated groups with the *M. haemolytica* isolates, all the infected mice groups with the *P. multocida* isolates dead within 24 h post the intra-peritoneal inoculation. *M. haemolytica* had no pathogenic effect although it was cleared from the lungs within 24 h. Heart blood impression smears proved characteristic of Pasteurellaceae species on Leishman's stain staining. The isolates showed typical morphological and cultural characteristics of dew drops, mucoid, haemolytic (*M. haemolytica*) and non haemolytic colonies (*P. multocida*) in blood agar. Culture smears shown characteristic Gram negative coccobacilli.

Table 2: Results of biochemical tests for *M. haemolytica* and *P. multocida*

Biochemical tests	<i>Mannheimia haemolytica</i>	<i>Pasteurella multocida</i>
Glucose	+	+
Sucrose	+	+
Fructose	+	+
Lactose	+	-
Silicin	+	-
Oxidase	+	+
Catalase	+	+
Urease production	-	-
Nitrate reduction tests	+	+
Indole	-	+
Voges-proskauer	-	-
Citrate	-	-
Methyl red	-	-

+: Positive, -: Negative

Fig. 1(a-b): (a) M: 50 bp ladder, lane 1-3 RAPD patterns of OPA-4 for *M. haemolytica* and (b) M: 50 bp ladder, lane 1-4 RAPD patterns of OPA-4 for *P. multocida*

Fig. 2(a-b): (a) M: 50 bp ladder, lane 1-3 RAPD patterns of OPA-9 for *M. haemolytica* and (b) M: 50 bp ladder, lane 1-4 RAPD patterns of OPA-9 for *P. multocida*

Molecular studies results: The profile generated by RAPD analysis using OPA-4 marker for *M. haemolytica* and *P. multocida* illustrates in Fig. 1a and b. *M. haemolytica* isolates observed with different RAPD patterns for each isolate. The difference was in the numbers of the amplified DNA fragments and the amplified DNA fragments molecular size. *P. multocida* showed two different RAPD patterns (Fig. 1b). The genetic variation between the two species was observed in the numbers of the amplified DNA fragments and the DNA fragments molecular size. The total numbers of the amplified DNA fragments for *M. haemolytica* ranged from 3-7 and the molecular sizes varied between 150-1500 bp compared to 5-6 and 300-900 bp, respectively for *P. multocida* isolates.

The profile generated by RAPD analysis using OPA-9 marker for *M. haemolytica* and *P. multocida* illustrates in

Fig. 2a and b. *M. haemolytica* and *P. multocida* isolates observed with different RAPD patterns for each isolate. The amplified DNA fragment size at 700 bp was remarkable fragment for both species however the total numbers of the amplified DNA fragments and the molecular sizes between the isolates for each species were difference. The total numbers of the amplified DNA fragments for *M. haemolytica* ranged from 2-9 and the molecular sizes varied between 200-800 bp compared to 1-5 and 250-1500 bp, respectively for *P. multocida* isolates.

The profile generated by RAPD analysis using OPA-20 marker for *M. haemolytica* and *P. multocida* illustrates in Fig. 3a and b. *M. haemolytica* isolates observed with different RAPD patterns for each isolate. The difference was in the numbers of the amplified DNA fragments and the DNA

Fig. 3(a-b): (a) M: 50 bp ladder, lane 1-3 RAPD patterns of OPA-20 for *M. haemolytica* and (b) M: 50 bp ladder, lane 1-4 RAPD patterns of OPA-20 for *P. multocida*

Fig. 4(a-b): (a) M: 50 bp ladder, lane 1-3 RAPD patterns of OPC-5 for *M. haemolytica* and (b) M: 50 bp ladder, lane 1-4 RAPD patterns of OPC-5 for *P. multocida*

fragments molecular size. *P. multocida* showed two different RAPD patterns (Fig. 3b). The total numbers of the amplified DNA fragments for *M. haemolytica* ranged from 1-4 and the molecular sizes varied between 350-1500 bp compared to 3-5 and 300-900 bp, respectively for *P. multocida* isolates.

The profile generated by RAPD analysis using OPC-5 marker for *M. haemolytica* and *P. multocida* illustrates in Fig. 4a and b. *M. haemolytica* isolates observed with different RAPD patterns for each isolate. The difference was in the numbers of amplified DNA fragments and the amplified DNA fragments molecular size. *P. multocida* showed three different RAPD patterns between the four isolates (Fig. 4b). The total numbers of the amplified DNA fragments for *M. haemolytica* ranged from 3-6 and the molecular sizes varied between 200-1000 bp compared to 3-5 and 450-900 bp, respectively for *P. multocida* isolates.

The ERIC marker's pattern for *M. haemolytica* and *P. multocida* shows in Fig. 5a and b. The ERIC molecular pattern size was difference between the two species. The amplified DNA fragments molecular size of the ERIC pattern for *M. haemolytica* ranged from 75-1000 bp compared to 100-325 bp for *P. multocida*.

DISCUSSION

The different isolates of *M. haemolytica* were observed heteromorphic RAPD patterns for all markers used except OPA-20 marker which was monomorphic in isolate 3 (Fig. 3a). The used markers showed 1-9 amplified fragments ranged from 75-1500 bp. The results illustrated that the isolates represented different genetic heterogeneity strains. The numbers of the amplified fragments obtained were in

Fig. 5(a-b): (a) M: 50 bp ladder, lane 1-3 ERIC and patterns for *M. haemolytica* (b) M: 50 bp ladder, lane 1-4 ERIC patterns for *P. multocida*

disagreement with the results reported by Saad *et al.*¹⁹ and Hawari *et al.*²⁰. The variation in the results could be traced to the usage of more and different markers than those in their study. The achieved results could be used for further studies to correlate between the virulent variation of *M. haemolytica* and the molecular distinctive patterns.

The results of the RAPD markers patterns for *P. multocida* different isolates were heteromorphic for all markers used except OPA-9 marker which was monomorphic in isolate 3 (Fig. 2b). The results of heteromorphic RAPD patterns of the *P. multocida* were in agreement with previous studies results that used different RAPD markers than those used in the study¹⁵⁻²⁰. The results of the amplified fragments for each marker in the different isolates revealed that the *P. multocida* genome was genetically similar between two out of 4 isolates however the genetic similarity was not repeated between the same isolates in all markers used (Fig. 1b-4b). The obtained results could be used for further studies to correlate between the virulent variations of the *P. multocida* and the molecular distinctive patterns.

Comparative the results of the RAPD patterns between *M. haemolytica* and *P. multocida* had detected the differences in the numbers and the molecular sizes of the amplified DNA fragments. The amplification of OPA-9 marker showed the highest numbers of DNA fragments from *M. haemolytica* genome compared to OPA-4 marker which was amplified with highest fragments numbers from *P. multocida* genome. The DNA fragments size generated by different RAPD markers ranged from 150-1500 bp in *M. haemolytica* compared to 250-1500 bp ranged for *P. multocida*. The results investigated that the RAPD markers have molecular characteristic differences between

M. haemolytica and *P. multocida*. The determined results revealed that the *M. haemolytica* genome has highly genetic heterogeneity compared to *P. multocida* genome which showed similarity between two isolates out of 4 isolates (Fig. 1a-4a and 1b-4b). The study results were in disagreement with the results reported by Saad *et al.*¹⁹ and Hawari *et al.*²⁰. Their studies reported that the *M. haemolytica* genome was low in genetic heterogeneity compared to the *P. multocida* genome. The difference in the results between the present study and earlier studies could be due to the use of different RAPD markers than those used in earlier study. However, the present study and earlier studies are in agreement that the RAPD markers can be used to clearly distinguish between the two members of the Pasteurellaceae family.

The fingerprint induced by ERIC marker observed with 6 bands ranged from 75-1000 bp in *M. haemolytica* while it was ranged from 100-325 bp with 5 bands in *P. multocida*. The amplified DNA fragments molecular sizes of the ERIC pattern for *M. haemolytica* were similar for all isolates. The same observation was recorded for *P. multocida*. The fingerprints study resulted from *M. haemolytica* strains using ERIC marker is considered the first report. The obtained results pattern in all isolates for both species is related to that ERIC marker depends on the highly conserved central inverted repeats location in the bacterial genome^{9,21}. The molecular sizes of *P. multocida* ERIC patterns in the study were in agreement with the molecular sizes of the ERIC patterns obtained by Leotta *et al.*²¹ and Ahmed *et al.*²² in different *P. multocida* strains. The results suggested that the ERIC-PCR is reliable and rapid molecular tool to identification and differentiation between Pasteurellaceae family (*M. haemolytica* and *P. multocida*).

Comparative results of RAPD markers and ERIC marker in *M. haemolytica* and *P. multocida* revealed that the RAPD markers patterns were variables between the different isolates in both species while ERIC reported the same molecular patterns for all isolates whether in *M. haemolytica* or *P. multocida*. The results suggested that ERIC marker can be used to differentiation between the different strains of the two species.

CONCLUSION

The obtained results of RAPD markers could be useful for further study to correlate between the variation of the bacterial strains virulence and the heteromorphic distinctive patterns. ERIC marker is reliable and rapid molecular tool for characterization and differentiation between the Pasteurellaceae family (*M. haemolytica* and *P. multocida*) that can be used as diagnostic tools.

SIGNIFICANCE STATEMENT

The study demonstrated the possibility using of RAPD and ERIC markers as rapid and accurate advanced molecular tools to characterization and differentiation between *P. multocida* and *M. haemolytica* that can be useful in the rapid diagnosis of Pasteurellosis. The study will help researchers in saving the time and the hard work from using traditional methods for the characterization and differentiation between *P. multocida* and *M. haemolytica*. Thus, the study is pioneering and unique in its reach.

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