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Research Article
Molecular Characterization of *Pasteurella multocida* Isolated from Rabbit in Egypt

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Abstract

Objective: The study aimed to molecular characterization of *Pasteurella multocida* local strain using Enterobacterial Repetitive Intergenic Consensus (ERIC), 13 virulence genes and the outer membrane protein (*OmpH*) gene. Also, development of diagnostic markers by multiplex PCR from investigated genes. **Methodology:** The ERIC-PCR was used for the detection of the finger print of *Pasteurella multocida* local strain isolated from rabbits. Polymerase Chain Reaction (PCR) with specific primers of the virulence genes and *OmpH* gene was used for molecular characterization of *Pasteurella multocida* local strain. The multiplex-PCR protocol was used to develop diagnostic genes markers. **Results:** *Pasteurella multocida* ERIC pattern was identified as seven DNA bands ranging from less than 100-850 bp. The results determined the molecular sizes of *toxA*, *pfhA*, *soda*, *nanB*, *nanH*, *fimA*, *hsf-1*, *hsf-2*, *hgbA*, *hgbB*, *ptfA*, *oma87*, *tbpA* and *OmpH* genes. Four multiplex-PCR were configured from investigated genes. Multiplex 1 amplified both of *hgbB* and *oma87* genes. Multiplex 2 included *fimA* and *ptfA* genes. Multiplex 3 used *nanB* and *hgbB* genes. Multiplex 4 amplified *nanB* and *ptfA* genes. **Conclusion:** Molecular characterization of *P. multocida* local strain using Enterobacterial Repetitive Intergenic Consensus (ERIC), 13 virulence genes and the outer membrane protein (*OmpH*) gene provided data information that could be used as diagnostic tool, epidemiological markers and in vaccine development.

Key words: *Pasteurella multocida*, PCR, multiplex-PCR, virulence genes, ERIC

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Pasteurella multocida is a common bacterial disease contributes to heavy losses in the animal production as well as a hazardous threat to human health. It is associated with haemorrhagic septicaemia in cattle and buffaloes, pneumonic pasteurellosis in sheep and goats, fowl cholera in poultry, snuffles in rabbits and cellulitis and septicemia in humans[1].

Advanced molecular biology techniques become a very useful approach for detecting microbes universally. Stahel *et al.*[2] proved that the REP-PCR typing represents a suitable tool for genetic characterization of rabbit Pasteurellaceae isolates while the biochemical analysis showed high heterogeneity and in some cases provided unclear results.

The wide host of *P. multocida* and different courses of infection lead to the need for extensive studies concerning the prevalence and distribution of capsule and virulence genes. The distribution of virulence genes of *P. multocida* in different animals has been studied by Ewers *et al.*[3] Their study recorded a high prevalence of the toxin gene among the strains from small ruminants.

Development of the multiplex-PCR protocol depends on the molecular characterization of *P. multocida* strains has been successfully used as a useful tool for rapid and simultaneous detection of virulence genes[4,5]. The significance of the molecular characterization of *P. multocida* strains confirmed by Gautier *et al.*[6], Sellyei *et al.*[7], Prabhakar *et al.*[8], Ferreira *et al.*[9] and Verma *et al.*[10] studies. They clarified that the epidemiological data together with molecular characteristics of individual strains could help to design and implement adequate preventive and intervention strategies.

In Egypt, *Pasteurella multocida* is a common pathogen in rabbits causing series outbreaks and considerable economic losses in rabbit industry[11-13]. The study aimed: (1) To molecular characterization of *P. multocida* isolated from rabbit using Enterobacterial Repetitive Intergenic Consensus (ERIC) and specific virulence genes of *P. multocida* and (2) To develop a *P. multocida* diagnostic tool by a multiplex-PCR.

MATERIALS AND METHODS

Samples: The samples were collected by sterile swabs from external nares of healthy and suspected infected rabbits. Each swab was plated on tryptic soy agar (Oxoid), supplemented with 5% sheep blood[14]. The isolates were subjected to further identification using Gram staining and standard biochemical procedures according to MacFaddin[15].

***Pasteurella multocida* DNA extraction:** Bacterial genomic DNA was extracted according to Ozbey *et al.*[16].

Molecular characterization of *P. multocida* genes: Polymerase chain reaction with specific primers for Enterobacterial Repetitive Intergenic Consensus (ERIC), 13 virulence genes and the outer membrane protein (*OmpH*) gene was used.

The primers sequences of enterobacterial repetitive intergenic consensus was according to Amonsin *et al.*[17]. The PCR program was denaturation at 95°C, 10 min, then 30 cycles of denaturation at 94°C for 1 min, annealing temperature at 52°C for 1 min, extension at 65°C for 8 min and a final extension at 65°C for 16 min.

The specific primers of 13 genes studied were obtained from available scientific research articles (Table 1). The *OmpH* primers were deduced in this study from the GenBank sequencing data [F: GCG TTT CAT TCA AAG CAT CTC, R: TTT AGA TTG TGC GTA GTC AAC]. The optimized PCR program was denaturation at 95°C: 5 min, 35 cycles of denaturation at 94°C: 45 sec, annealing temperature (Table 1) 30 sec, 72°C: 30 sec, extension at 72°C for 7 min.

Multiplex-PCR was developed (Table 2) from investigated genes in this study. The optimized PCR program was denaturation at 95°C: 5 min, 30 cycles of denaturation at 94°C: 30 sec, annealing 56°C, 30 sec, 72°C: 30 sec, extension at 72°C for 7 min. For each amplification reaction, a mixture of reagents (master mix) and primers (total volume 25 µL) were prepared.

The PCR products were electrophoresed on 1% agarose gel (Invitrogen Ultrapure™ Agrose®-Carlsbad, USA) together with a 100 bp DNA ladder (Promega Corporation, France) for molecular weight estimation. The amplified products were visualized in an ultraviolet light transilluminator, photographed and analyze.

RESULTS

The result of *P. multocida* ERIC electrophoresis patterns showed seven DNA bands there sizes were less than 100, 100, 175, 280, 390, 710 and 850 bp (Fig. 1).

Thirteen virulence genes (*toxA*, *pfhA*, *sodaA*, *nanB*, *nanH*, *fimA*, *hsf-1*, *hsf-2*, *hgbA*, *hgbB*, *ptfA*, *oma87* and *tbpA*) and the outer membrane protein (*OmpH*) gene were identified. The fragments size of the successful PCR amplified products of the tested genes are recorded in Table 1 and Fig. 2-7.

Four multiplex-PCR were configured from investigated genes. Multiplex 1 amplified both o f *hgbB* and *oma87* genes, multiplex 2 included *fimA* and *ptfA* genes, multiplex 3 used *nanB* and *hgbB* genes and multiplex 4 amplified *nanB* and *ptfA* genes. The developed multiplex-PCR fragments are shown in Fig. 8-11.

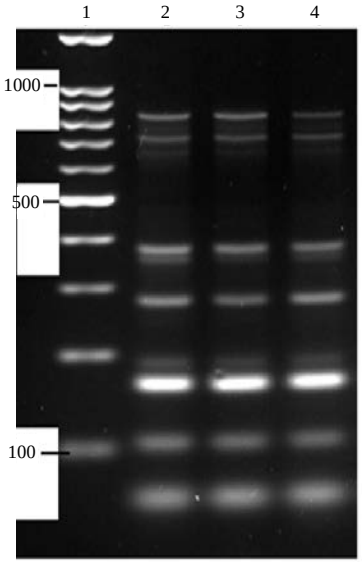

Fig. 1: Agarose gel electrophoreses Lane 1: A 100 bp ladder, Lane 2-4: Enterobacterial Repetitive Intergenic Consensus (ERIC)

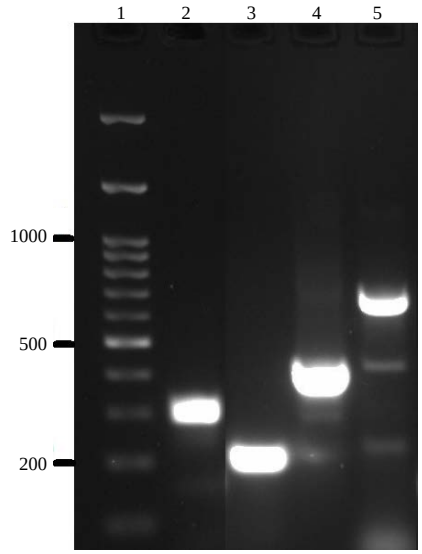

Fig. 2: Agarose gel electrophoreses of DNA fragments obtained with specific primers Lane 1: A 100 bp ladder, Lane 2: *phfA*, Lane 3: *ptfA*, Lane 4: *fimA* and Lane 5: *hsf-2* genes

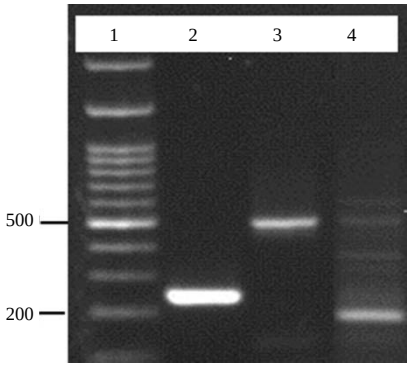

Fig. 3: Agarose gel electrophoreses of DNA fragments obtained with specific primers Lane 1: A 100 bp ladder, Lane 2: *nanH*, Lane 3: *hsf-1* and Lane 4: *tbpA* genes

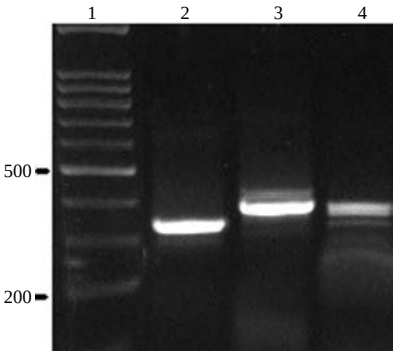

Fig. 4: Agarose gel electrophoreses of DNA fragments obtained with specific primers Lane 1: A 100 bp ladder, Lane 2: *hgbA*, Lane 3: *nanB* and Lane 4: *oma87* genes

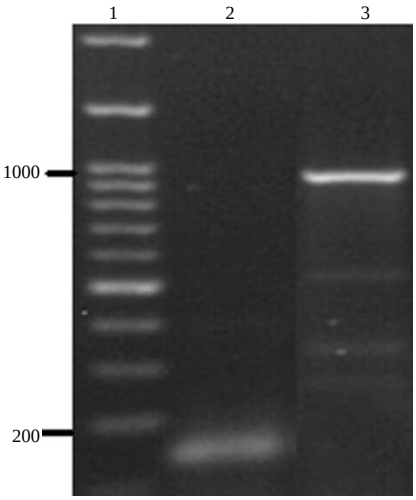

Fig. 5: Agarose gel electrophoreses of DNA fragments obtained with specific primers Lane 1: A 100 bp ladder, Lane 2: *hgbB* and Lane 3: *toxA* genes

Table 1: Optimized annealing temperatures and the amplified DNAs size of fourteen specific genes of *P. multocida*

Genes	Annealing temperature (°C)	References	Amplified size (bp)
Dermonecrotoxin (*toxA*)	56	Ewers *et al.*[3]	864
Filamentous hemagglutinin (*pfhA*)	56	Ewers *et al.*[3]	300
Superoxid dismutases (*soda*)	60	Ferreira *et al.*[9]	500
Neuraminidase-B *(nanB)*	56	Ewers *et al.*[3]	380
Neuraminidase-H (*nanH*)	58	Ewers *et al.*[3]	250
Filamentous hemagglutinin (*fimA*)	56	Tang *et al.*[18]	400
Autotransporteradhesins-1 (*hsf-1*)	58	Tang *et al.*[18]	500
Autotransporteradhesins-2 *(hsf-2)*	56	Tang *et al.*[18]	700
Iron acquisition related factors-A (*hgbA*)	56	Ewers *et al.*[3]	320
Iron acquisition related factors-B (*hgbB*)	56	Ewers *et al.*[3]	150
Subunit of type 4 fimbriae (*ptfA*)	56	Tang *et al.*[18]	220
Outer membrane protein (*oma87*)	56	Ewers *et al.*[3]	360
Transferrin binding protein encoding (*tbpA*)	57	Ewers *et al.*[3]	200
Outer membrane protein H (*OmpH*)	60	This study	467

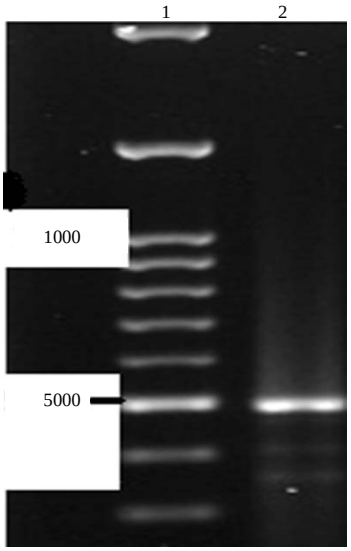

Fig. 6: Agarose gel electrophoreses of DNA obtained with specific primers Lane1: 100 bp ladder and Lane 2: *sodA*

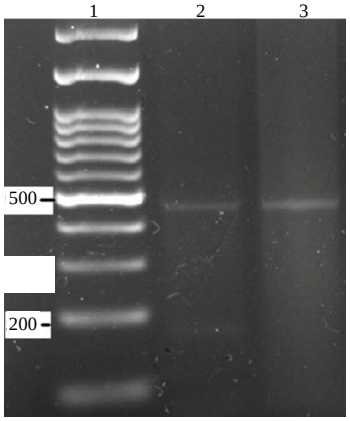

Fig. 7: Agarose gel electrophoreses of DNA fragments obtained with specific primers Lane 1: A 100 bp ladder and Lane 2-3: *OmpH* gene

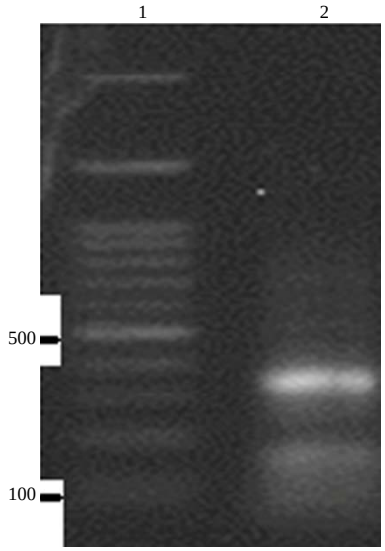

Fig. 8: Agarose gel electrophoreses of DNA fragments obtained with specific primers Lane 1: A 100 bp ladder and Lane 2: *hgbB* and *oma87* genes as multiplex 1

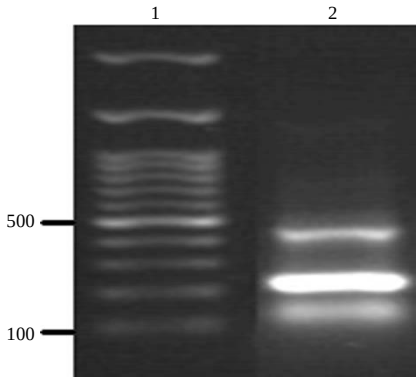

Fig. 9: Agarose gel electrophoreses of DNA fragments obtained with specific primers Lane 1: A 100 bp ladder and Lane 2: *fimA* and *ptfA* genes as multiplex 2

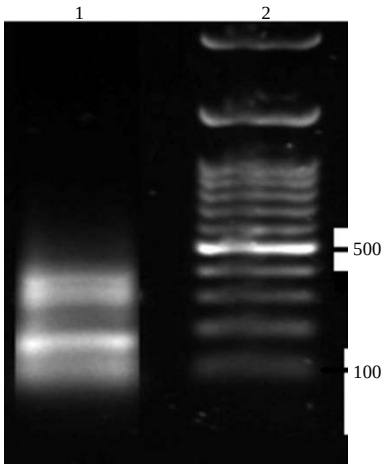

Fig. 10: Agarose gel electrophoreses of DNA fragments obtained with specific primers Lane 1: *nanB* and *hgbB* genes as multiplex 3 and Lane 2: A 100 bp ladder

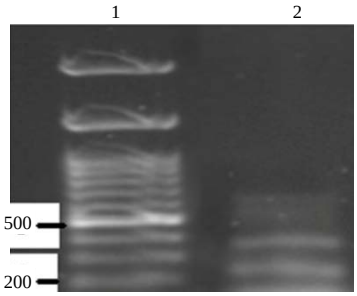

Fig. 11: Agarose gel electrophoreses of DNA fragments obtained with specific primers Lane 1: A 100 bp ladder and Lane 2: *nanB* and *ptfA* genes as multiplex 4

Table 2: Multiplex-PCR for *P. multocida* diagnoses

Multiplex-PCR	Virulence-associated genes	Expected size of amplified DNA (bp)
1	*hgbB*, *oma87*	150 and 360
2	*fimA*, *ptfA*	400 and 220
3	*nanB*, *hgbB*	380 and 150
4	*nanB*, *ptfA*	380 and 220

DISCUSSION

The ERIC typing has been identified as a tool for *Pasteurella* genetic characterization and it used extensively for bacterial differentiation, rapid epidemiological analysis and determination of outbreak-related strains[16,19]. In this study, to generate DNA fingerprint for *P. multocida* the ERIC marker was used. The obtained result was different from previously published studies[19,2] used ERIC-PCR for characterization of *P. multocida* local strain, that difference could be related to the specificity of the *P. multocida* strains.

The investigated genes in this study play an important role in epidemiological and pathogenesis effect of *P. multocida*[3,20,18,7,8]. The previous study by Shayegh *et al*.[20], Prabhakar *et al*.[8] and Verma *et al*.[10] recommended using *tbpA*, *phfA* and *ptfA* genes as an epidemiological marker. The *fimA*, *hsf-2*, *hgbA*, *nanB* and *oma87* genes of *P. multocida* recognized as virulence factors responsible for the pathogenicity in the target host[21,22,5]. The *hgbB* and *toxA* genes are virulence genes used for detection of the *P. multocida* pathogenicity and the multiplex-PCR methodology for disease diagnosis[5]. The studies related to the *sodA* sequences showed higher divergence than the corresponding 16S rRNA genes which makes it as a potent target to differentiate related species[9,10]. The results of this study revealed that the amplified PCR products of tested genes of *P. multocida* isolated from Egyptian rabbits showed differences in genes fragments sizes amplifications compared to several previous studies which used the same genes loci isolated from different animals (cattle, buffalo, pig and sheep)[3,13].

The *OmpH* gene is reported as surface-exposed conserved immunodominant porin[23]. In this study, the specific primers for *OmpH* gene was developed using the GenBank sequencing data. Development of these primers aimed to obtain specific primers from the conservative gene of *P. multocida*. Accordingly, these primers could be considered as a diagnostic tool reference for the *P. multocida* local strain.

Multiplex-PCR is a rapid and reliable technique to determine multiple genes of microorganisms for diseases diagnosis, reducing the amount of reagents used and time required[4,15]. The results of tested genes were used to develop a multiplex-PCR for diseases diagnosis induced by *P. multocida* local strain (Table 2). Optimization of the multiplex-PCR protocols developed in this study depends on the same primers annealing conditions without interfering with one another in the multiplex-PCR and to the different in DNA bands produced. The results confirmed the significance study of molecular characterization of *P. multocida* local strain.

Control of the disease outbreaks is dependent upon the system of monitoring, early detection and vaccination. Definitive diagnosis of bacterial or viral disease requires detection of the microorganism, antigen or genome in clinical material. Traditional microbiological methods for detection of pathogens can be slow, are not sensitive, may not distinguish infection from colonization and are influenced by previous antibiotic therapy[24,25]. As each bacterial strain has a specificity at the molecular level.

CONCLUSION

The molecular characterization of *P. multocida* local strain in rabbits investigated for the study using Enterobacterial Repetitive Intergenic Consensus (ERIC), 13 virulence genes and the outer membrane protein (*OmpH*) gene provided data information that could be used as diagnostic tool, epidemiological markers and in vaccine development.

ACKNOWLEDGMENT

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SIGNIFICANCE STATEMENT

Molecular characterization of the indigenous pathogen is required for diagnosis, epidemiological study, vaccine development and detection any shift or mutation of the microorganism genomic as the local vaccine strain should be prepared from the circulating strain in the region.

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