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# Rapid diagnosis of virulent *Pasteurella multocida* isolated from farm animals with clinical manifestation of pneumonia respiratory infection using 16S rDNA and *KMT1* gene

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ABSTRACT

**Objective:** To characterize intra-isolates variation between clinical isolates of *Pasteurella multocida* (*P. multocida*) isolated from sheep, cattle and buffalo at molecular level to check the distribution of pneumonia and hemorrhagic septicemia in some regions of Fayoum, Egypt.
**Methods:** These isolates were obtained from various locations in the Fayoum Governorate, Egypt and they were identified by amplifying 16S rDNA and *KMT1* genes using their DNA as a template in PCR reaction.
**Results:** The results demonstrated that the five selective isolates of *P. multocida* had similar size of PCR products that generated one band of 16S rDNA having 1 471 bp and *KMT1* gene having 460 bp. The phylogenetic tree and similarity of the five selective isolates of *P. multocida* which were collected from GenBank database were calculated and analyzed for the nucleotide sequence of 16S rDNA and *KMT1* genes. The sequencing result of 16S rRNA gene product (1 471 bp) for the five selective isolates of *P. multocida* showed that the isolates of sheep (FUP2) shared 94.08%, 88.10% homology with the buffalo isolate (FUP8) and cattle isolate (FUP9) respectively, whereas, the buffalo isolate (FUP5) shared 98.18% and 94.40% homology with the cattle isolates (FUP12 and FUP9).
**Conclusions:** The results indicated the relationships of *P. multocida* isolated from buffalo and cattle rather than the close relationships between *P. multocida* isolated from cattle and sheep. Diagnosis of *P. multocida* by 16S rDNA and *KMT1* gene sequences was important to determine the antigen that is responsible for protective cover within the same group of animals and to help for the production of new vaccines for the control of microbial infection for domestic animals.

## 1. Introduction

Sheep, buffalo and cattle represent an important standing in the domestic animal wealth, especially in the Middle East and Arab countries. Sheep represent an important member of the livestock and are kept mainly for meat and wool production, where they yield about 10.4% of meat consumption; therefore, a great attention should be directed toward caprine and ovine industry to meet the people's animal protein requirements[1]. Respiratory system disease is the leading cause of death in the lambs and kids and leads to decrease in the productivity of farm animals. The most common problem associated with the lower respiratory tract is pneumonia which could be occurred due to *Pasteurella multocida* (*P. multocida*). The prevalence of *P. multocida*, a cause of bovine respiratory disease, was studied in a random sample of dairy farms throughout Scotland. A higher prevalence was detected in dairy calves than beef calves[2]. Shayegh *et al*. showed that high prevalence of *toxA* and *tbpA* among goat is very similar to the ones in sheep isolates[3]. Comparison of virulence genes profile showed the possibility of *P. multocida* transmission between sheep and goat. In addition, the goat may serve as a reservoir of *Pasteurella* strains that are likely to be virulent in bighorn sheep. Considering the very close relation and nose-to-nose contact between sheep and goat, it is thought that *P. multocida* may be introduced to the domestic sheep population through domestic goats[4]. The disadvantage of conventional technique for Pasteurellaceae identification includes many biochemical and enzymatic tests, required to long time and the results of phenotypic identification are sometimes unreliable or disappointing due to potential variation of their results according to the analytical methods and the chemicals purity, and also different according to the host of bacterial isolates[5]. On the other hand, the molecular diagnosis of *Pasteurella* spp. isolates includes analysis of nucleotide sequences of the 16S rRNA and *KMT1* genes have been overcome the disadvantage and limitation of phenotypic diagnosis

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and become a favorable technique in many international laboratories for identification and calculating the phylogenetic tree between Pasteurellaceae family as well as on the *P. multocida* subspecies level because of these advantages, reducing the time consumption, direct detection of organisms from clinical sample's genome, and the sensitivity and specificity of the diagnosis[6]. The 16S rRNA gene, a molecular marker for the identification of bacterial species, can be used for the identification of organisms at any level just by using a specific primer sequence[7].

Christensen and Bisgaard showed that *Pasteurella langaa* and *Pasteurella anatis* were unrelated to the avian group of *Pasteurella* using the sequences and phylogenetic analysis of the amplification of 16S rRNA gene obtained from PCR assay[8]. Jabbari *et al*. demonstrated the role of goat as a reservoir for *P. multocida* to sheep independent of *toxA* genes transmission using primers derived from conserved part of 16S-23S rRNA gene and DNA template isolated from nine avian *P. multocida* in modified PCR reaction[9]. The molecular weight of the clone *KMT1* gene was determined by Prabhakar *et al*. using specific PCR and the size of the PCR product were determined to be 866 nucleotides[10].

Deressa *et al*. reported that *P. multocida* and *Pasteurella haemolytica* can be detected directly from clinical and section tissues of acute respiratory disease of sheep using PCR protocol[11]. The results showed that both bacterial species gave positive reaction with specific amplification. Güler *et al*. found that the molecular weight of the *KMT1* gene product was amplified using multiplex PCR about 460 bp and they also reported that all bacterial isolates confirmed as *P. multocida* had *KMT1* gene with molecular weight about 460 bp[12].

The molecular characterization can be used for early detection and precise discrimination of the phylogeny of fastidious pathogens as well as virulence genes as a future step for vaccine preparation and it would ultimately contribute in establishing futuristic approach for controlling of this pathogenic organism. An alternative approach to control pathway could be the development of the specific subunit vaccine of disease depending on bacterial virulence genes sequencing. High-confidence single nucleotide polymorphisms will be used to design new (high-confidence single nucleotide polymorphisms) arrays to study variation across strains[13]. Therefore, the present study was conducted to characterize intra-isolates variation between clinical isolates of *P. multocida* at molecular level to check the distribution of pneumonia and hemorrhagic septicemia in some regions of Fayoum, Egypt. Also, the study is focusing on the characterizations of clinical isolates of *P. multocida* isolated from sheep, buffalo and cattle and identified by amplified 16S rDNA and *KMT1* genes using their DNA as a template in PCR reaction.

## 2. Materials and methods

### 2.1. Samples collection and isolates

A total of 12 isolates used in this study were isolated from private animals farms in Fayoum, Egypt (sheep: $n = 4$, cattle: $n = 4$ and buffalo: $n = 4$) (Table 1). These animals had a clinical manifestation of pneumonia. The nasopharyngeal swabs method was used to obtain the samples, and the samples were transported to the bacteriological laboratory, plated onto 10% sheep blood agar and incubated at 37 ℃ overnight for culturing and microbiological examination.

**Table 1**
Isolates of *Pasteurella* spp. used in the present study and their origin.

| Isolates codes | Source of isolates | Species |
|---|---|---|
| FUP1 | Sheep | *Pasteurella* spp. |
| FUP2 | Sheep | *Pasteurella* spp. |
| FUP3 | Sheep | *Pasteurella* spp. |
| FUP4 | Sheep | *Pasteurella* spp. |
| FUP5 | Buffalo | *Pasteurella* spp. |
| FUP6 | Buffalo | *Pasteurella* spp. |
| FUP7 | Buffalo | *Pasteurella* spp. |
| FUP8 | Buffalo | *Pasteurella* spp. |
| FUP9 | Cattle | *Pasteurella* spp. |
| FUP10 | Cattle | *Pasteurella* spp. |
| FUP11 | Cattle | *Pasteurella* spp. |
| FUP12 | Cattle | *Pasteurella* spp. |

### 2.2. Ethical approval

All the procedures were carried out in accordance with the guidelines laid down by Fayoum University and in accordance with local laws and regulations.

### 2.3. DNA extraction of P. multocida isolates

Genomic DNA isolation was carried out according to Ozbey *et al*. with some modification as follows: single colony was inoculated in brain heart infusion broth and grown overnight at 37 ℃ in shaking incubator[14]. About 1 mL of each culture was sedimented by centrifugation at 12 000 r/min for 10 min at room temperature. Then 300 μL of distilled water was added to the vortex tubes. The samples were incubated for 10 min at 56 ℃ and then 300 μL of K-buffer and 200 μg/mL of proteinase-K were added following 10 min boiling. The suspension was deprotonated by adding equal volume of phenol and mixed by hand for 5 min, then it was centrifuged at 11 600 r/min for 10 min. The DNA contained in the aqueous phase was precipitated with 40 μL of 0.3 mol/L sodium acetate, pH 7.0 and 1 mL of absolute ethanol for 1 h at -20 ℃. The mixture was centrifuged at 11 600 r/min for 5 min and the upper phase was discarded. The pellet was washed by 70% ethanol and centrifuged for 5 min, and the pellet was also dried and suspended in 50 μL of sterile distilled water and stored frozen at -20 ℃ until further studies.

### 2.4. PCR amplification of 16S rDNA gene

To confirm the species of *Pasteurella* isolates at the molecular level, the 16S rRNA genes of the five selective isolates of *Pasteurella* spp. were amplified by PCR using the universal primers (forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer: 5'-CTTGTGCGGGCCCCCGTCAATTC-3') according to Srivastava *et al*.[15]. The PCR was performed in the thermal cycler 2720 (Applied Biosystems, USA) in a total volume of 25 μL containing 1 μL of each primer, 200 μmol/L from the four deoxy-ribonucleoside triphosphates (dNTP), 5 μL of 10× PCR buffer, 1 μL of 25 mmol/L $MgCl_2$, 1 μL of template DNA, 1 μL of *Taq* DNA polymerase and 14.5 μL of PCR $H_2O$. The amplification programme consisted of an initial denaturation step at 94 ℃ for 5 min followed by 35 cycles of 1 min denaturation at 94 ℃, 1 min primer annealing at 42 ℃, 2 min extension at 72 ℃ and a final extension of 10 min at 72 ℃. About 8 μL of the final product for

each samples were analysed by electrophoresis on a 1.8% agarose gel, previously stained with ethidium bromide (0.2 μg/mL) and were run at 120 V for 1 h. The molecular marker used was a 100 bp ladder and the amplicons were observed under UV radiation.

### 2.5. Sequence analysis of the 16S rDNA gene

The PCR products were purified from mismatching PCR primers and dNTPs using montage PCR clean up kit (Millipore). The purified PCR products of approximately 1 471 bp were subjected to sequencing through technology services laboratory located in Korea and performed at Applied Biosystems model 3730 XL automated DNA sequencing system. The GenBank accession number for the 16S rRNA gene sequence of *Pasteurella* isolates was KR006971, KR006972, KR006973, KR006974 and KR006975.

### 2.6. Computational analysis (BLAST) and construction of phylogenetic tree

The data of the nucleotide sequence of the 16S rRNA gene obtained from the five selective *P. multocida* isolates were subjected to alignment with *P. multocida* sequences of the GenBank sequence database according to the method described by Hall[16]. MEGA version 3.1 program was used to generate the phylogenetic tree using the unweighted pair-group method with arithmetic means (UPGMA) method according to Kumar *et al*.[17].

### 2.7. PCR amplification of KMT1 gene

*KMT1* gene was amplified according to the method of Townsend *et al*. using KMTISP6 and KMT1T7 specific primers[18]. The PCR reaction was performed in the thermal cycler 2720 (Applied Biosystems, USA) in a total volume of 25 μL containing 1 μL of template DNA, 1 μL of each primer, 200 μmol/L from the four dNTPs, 5 μL of 10× PCR buffer, 1 μL of 50 mmol/L $MgCl_2$, and 1 μL of *Taq* DNA polymerase. The amplification programme consisted of an initial denaturation step at 94 ˚C for 5 min followed by 35 cycles of 1 min denaturation at 94 ˚C, 1 min primer annealing at 55 ˚C, 1 min extension at 72 ˚C and a final extension of 10 min at 72 ˚C. About 8 μL of aliquots of PCR products were checked by gel electrophoresis in 2% agarose gel and added ethidium bromide (0.5 μg/mL) to run buffer for visualization under UV transilluminator.

### 2.8. Sequence analysis of the KMT1 gene

The primers KMT1T7-KMT1SP6, previously designed for detection of *P. multocida*, were able to produce PCR product of approximately 460 bp. The PCR products were purified and subjected to sequencing through technology services laboratory located in Korea and performed at Applied Biosystems model 3730 XL automated DNA sequencing system. The GenBank accession number for the *KMT1* gene sequence of *Pasteurella* isolates was KR006976, KR006977, KR006978, KR006979 and KR006980.

### 2.9. Phylogenetic analysis

The phylogenetic tree for the data of the nucleotide sequence of the *KMT1* gene obtained from the five selective *P. multocida* isolates was subjected to sequences of *P. multocida* strains available at the public GenBank database according to Hall[16]. MEGA version 3.1 program was used to generate the phylogenetic tree using the UPGMA method according to Kumar *et al*.[17].

## 3. Results

### 3.1. Amplification of the 16S rDNA gene

Genomic DNA of the five selective isolates of *P. multocida* was analyzed by PCR amplification of 16S rDNA gene. Amplification of the 16S rDNA gene with universal primers L1 and EGE1 revealed single product estimated by agarose gel electrophoresis approximately 1471 bp, which was obtained from all the PCR amplification for the five selective isolates of *P. multocida* (Figure 1).

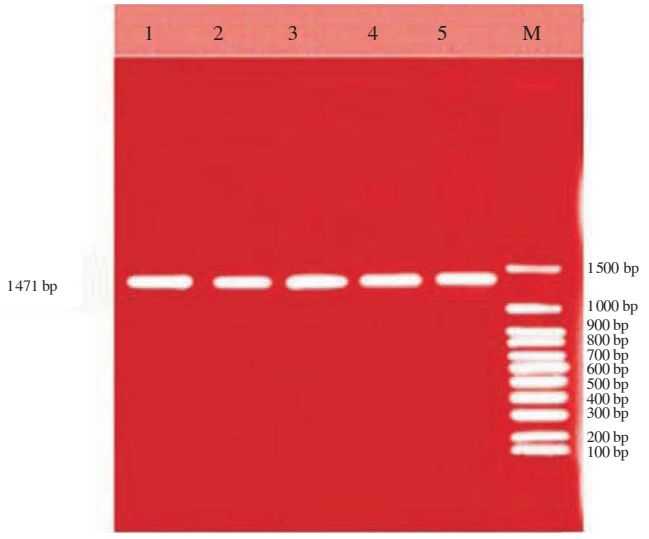


**Figure 1.** Agarose gel analysis of PCR products from amplification of 16S rDNA of five selective *P. multocida* strains.
Lane M: 100 bp DNA ladder; Lane 1: FUP2; Lane 2: FUP5; Lane 3: FUP8; Lane 4: FUP9; Lane 5: FUP12.

### 3.2. Phylogenetic analysis of the 16S rDNA sequences

Each sequence after editing was submitted to the GenBank and homology searches were done against the published *Pasteurella* sequence to compare with the related sequences in GenBank using BLAST and FASTA programs. Search results of each sequence giving the closest match to the test sample was used to determine the species of *Pasteurella* isolates. Multiple sequence alignment was carried out including 16S rDNA that indicated a similar sequence and the highest homology (98.44%) was found between *P. multocida* FUP5 and FUP8 isolated from buffalo. However, the less homology (87.60%) was found between *P. multocida* FUP2 from sheep and FUP12 from cattle (Table 2). Based on the results obtained from the five selective strains of *P. multocida*, the dendrogram was constructed from two primary genetic clusters as shown in Figure 2. The first cluster represented FUP2 which was isolated from sheep and the second cluster included two sub-clusters and the first included strain FUP9 from cattle. The other sub-cluster included strain FUP12 from cattle and the two strains isolated from buffalos (FUP5 and FUP8) were closely related in one linage, while the two strains from cattle FUP9 and FUP12 were highly distantly.

**Table 2**
Similarity coefficient percentage among the five selective *P. multocida* isolates based on 16Sr DNA sequence.

| Isolates | FUP2 | FUP5 | FUP8 | FUP9 | FUP12 |
|---|---|---|---|---|---|
| FUP2 | 100.00 | | | | |
| FUP5 | 88.10 | 100.00 | | | |
| FUP8 | 94.08 | 98.44 | 100.00 | | |
| FUP9 | 88.61 | 94.40 | 94.80 | 100.00 | |
| FUP12 | 87.60 | 98.18 | 97.60 | 94.10 | 100.00 |

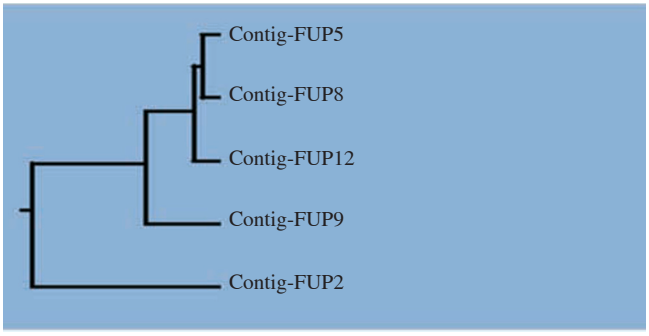


**Figure 2.** Phylogenetic tree showing the relationship between the five selective *P. multocida* strains based on 16S rDNA gene sequence.

For further reconfirmation and phylogenetic analysis of the strains, sequences were aligned using ClustalW (multiple sequence alignment) software tool according to the NCBI BLAST search against the GenBank sequence database and the isolates were designated as *P. multocida* (Table 3). The phylogenetic relationship of these isolates was presented in a distance-based rooted phylogentic tree (UPGMA) (Figure 3). The phylogenetic tree obtained by sequence analysis of 16S rDNA of the five selective strains of *P. multocida* and the seqences of 18 *P. multocida* strains obtained from GenBank database were represented in Figure 3. Bootstrap analysis with 1 000 bootstrap replications demonstrated two major branches and on the basis of the bootstrap values, the 24 *P. multocida* isolates were divided into different groups. The isolate (FUP8) was located with AY299305 *P. multocida*, the isolate (FUP5) was located with AF326324 *P. multocida* and the isolate (FUP9) was located with AY324032 *P. multocida* in same sub-cluster. On the other hand, the second sub-cluster included FUP8 and FUP12.

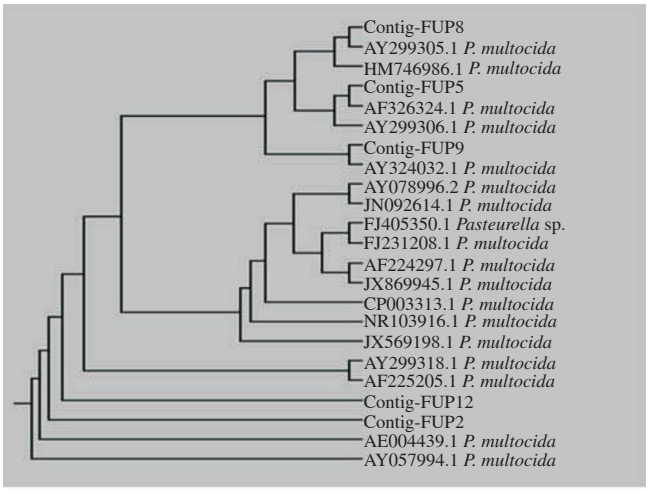


**Figure 3.** Phylogenetic tree showing the relationship between the five selective *P. multocida* strains and the closely related members of *Pasteurella* obtained from NCBI database according to 16S rDNA gene sequence.

### 3.3. PCR amplification of KMT1 gene

*P. multocida* species specific PCR assay was a suitable technique for specific detection of *P. multocida* compared with tradiontal bacteriological methods. In the present study, we used the primers KMT1SP6 and KMT1T7 to identify *P. multocida* by amplifying *KMT1* gene. All the five selective *P. multocida* isolates showed DNA band on gel approximately 460 bp (Figure 4).

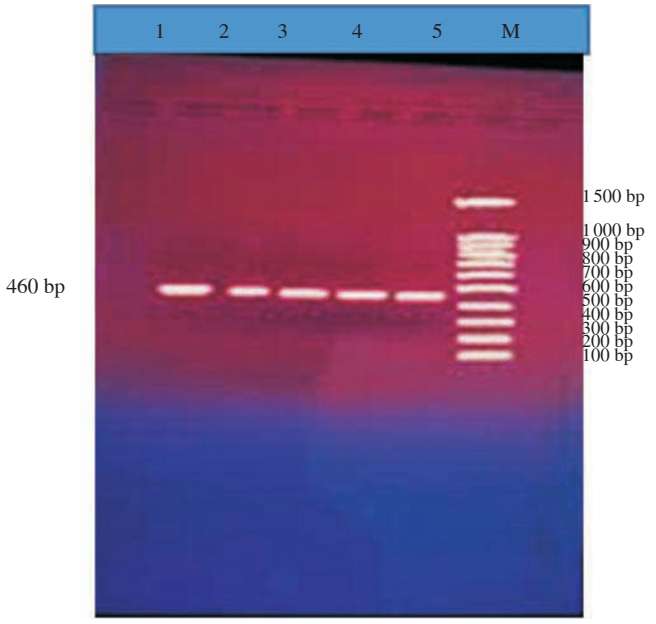


**Figure 4.** Agarose gel analysis of PCR products from amplification of *KMT1* gene of five selective *P. multocida* strains.
Lane M: 100 bp DNA ladder; Lane 1: FUP2; Lane 2: FUP5; Lane 3: FUP8; Line 4: FUP9; Lane 5: FUP12.

### 3.4. Phylogenetic analysis of the KMT1 gene sequence

Multiple sequence alignment and phylogenetic analysis of *KMT1* gene that was amplified from five selective *P. multocida* strains were carried out. The highest homology (99.12%) was found between *P. multocida* FUP5 and FUP8 from buffalo. However, the less homology (92.32%) was found between the different isolates (*P. multocida* FUP2 from sheep and FUP5 from buffalo) as shown in Table 3. Based on the results obtained from similarity among the five selective strains of *P. multocida*, the dendrogram was divided into two main clusters (Figure 5). The first cluster included FUP2 *P. multocida* isolated from sheep and the second cluster included two sub-clusters. The first included strains FUP5 and FUP8 which were isolated from buffalo and were closely related in one linage. The second included strains FUP9 and FUP12 from cattle, which were closely related in one linage.

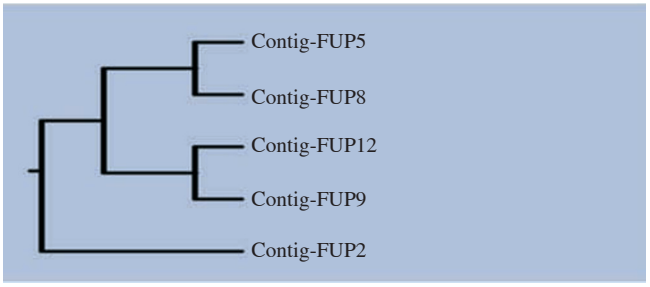


**Figure 5.** Phylogenetic tree showing the relationship between the five selective *P. multocida* strains based on *KMT1* gene sequence.

The phylogenetic tree obtained by sequence analysis of *KMT1* gene of the five selective *P. multocida* strains and the sequences of nine *P. multocida* strains which was obtained from the GenBank database were represented in Figure 6. The dendrogram was divided into two main clusters. The first cluster included two sub-clusters and the second included DQ233649 *P. multocida*. However, the second sub-cluster included the group of strains with accession No. AY225342, AY157572, DQ233648, AY225341 and AF016259 *P. multocida*. The second main cluster included two sub-clusters: the first included FUP2 and the second cluster included two sub-clusters. The first sub-cluster included strains with accession No. DQ871029 and DQ871028 *P. multocida*. However, the second sub-cluster included the group of FUP5, FUP8, FUP9, FUP12 and FJ986389 *P. multocida*.

**Table 3**
Similarity coefficient percentage among the five selective *P. multocida* isolates based on *KMT1* gene sequence.

| Isolates | FUP2 | FUP5 | FUP8 | FUP9 | FUP12 |
|---|---|---|---|---|---|
| FUP2 | 100.00 | | | | |
| FUP5 | 92.32 | 100.00 | | | |
| FUP8 | 92.54 | 99.12 | 100.00 | | |
| FUP9 | 92.98 | 97.81 | 97.81 | 100.00 | |
| FUP12 | 93.42 | 98.25 | 98.03 | 98.47 | 100.00 |

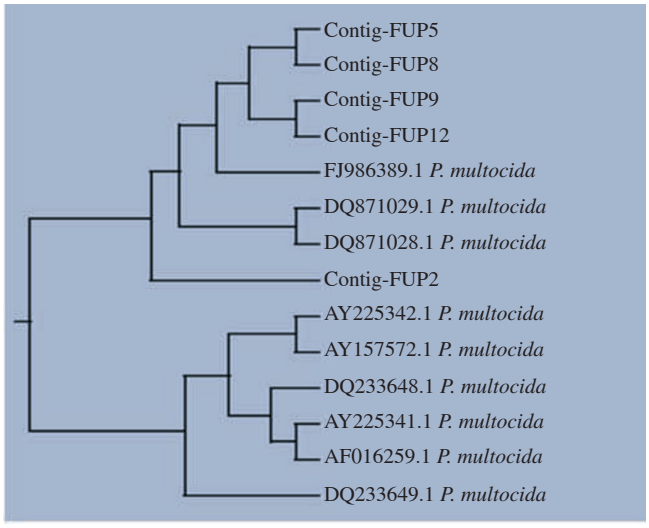


**Figure 6.** Phylogenetic tree showing the relationship between the five selective *P. multocida* strains and the closely related members of *Pasteurella* obtained from NCBI database according to *KMT1* gene sequence.

## 4. Discussion

The morphological and biochemical identification of *P. multocida* isolates should be confirmed by molecular identification based on 16S rRNA and *KMT1* genes analysis. Ribosomal DNA genes (16S rDNA) possess characteristics that are suitable for the identification of bacterial isolates at the species level. Partial 16S rRNA gene sequences for the five selective *Pasteurella* isolates were deposited in NCBI GenBank under GenBank accession numbers KR006971 to KR006975. Obtained results are in accordance with Dey *et al*.[19]. They investigated the phylogenetic relationships of five isolates of *P. multocida* serotype B belonging to buffalo, cattle, pig, sheep and goat by comparative sequence analysis of 16S rRNA gene. The isolates of cattle (PM75), pig (PM49) and sheep (PM82) shared 99.9% homology with the buffalo isolate (vaccine strain P52), whereas the goat isolate (PM86) shared 99.8% homology with the vaccine strain. Shayegh *et al*. recorded that all *P. multocida* isolates of goats except one with a variation site 99.9% shared 100% homology with the 16S rRNA gene sequence of *P. multocida* strain NCTC 10322 type A (subsp. *multocida*)[20]. On the other hand, all of the ovine isolates showed 99.9% homology with the same reference strain. The genetic diversity of 86 *P. multocida* strains isolated from ovine, bovine and avian was studied by Davies using the sequence analysis of the 16S rRNA genes and they isolated a fragment with molecular weight about 1 468 bp[21].

*P. multocida* specific PCR assay is a suitable technique for specific detection of *P. multocida* compared with traditional bacteriological methods. In this respect, we are using PM-PCR assay to identify the five selective *Pasteurella* isolates by amplifying the DNA fragment within *KMT1* gene according the method described by Townsend *et al*.[18]. Our results in this context are in agreement with the results obtained from the *P. multocida* strains which were isolated from sheep using the primers KMT1SP6 and KMT1T7 to identify the isolates[10,22]. Primers for *P. multocida* were designed to detect a fragment of the *KMT1* gene encoding the outer membrane protein. Primers specific to *Pasteurella canis*, *Pasteurella dagmatis* and *Pasteurella stomatis* were based on the manganese-dependent superoxide dismutase gene (*sodA*). The PCR assays were shown to be species-specific, providing a valuable supplement to phenotypic identification of species within this group of bacteria[23]. Munir *et al*. reported that the outer membrane protein represents the vital factor in the pathogenesis of pasteurellosis[24]. Also, using PM1 and PM2 primers, a 300 bp PCR product was obtained by Borowski *et al*.[25], with the D-type *P. multocida* strain. However, Liu *et al*. found that by using oligonucleotides primers and PCR, PM762F/R generated a 597 bp DNA fragment and primers PM1231F/R facilitates the amplification of a 601 bp[26]. Both primers sets formed specific PCR products of appropriate sizes of genomic DNA of *P. multocida* only. So, their results suggested that PM762F/R and PM1231F/R are species-specific genes in *P. multocida*. A multiplex PCR assay for simultaneously detecting four pathogenic bacteria in ducks was developed using target genes (*KMT1*, the invasion protein gene, 16S rDNA and alkaline phosphatase gene) for *P. multocida*, *Salmonella enterica*, *Riemerella anatipestifer* and *Escherichia coli* respectively[27].

Phenotypic characterization of *P. multocida* is undesirable due to the variation of biochemical and enzymatic assays. Therefore, molecular study conducted at the five selective *Pasteurella* isolates which is recovered from clinically sick of farm animals is more suitable for identification and characterization of *P. multocida* isolates and gives similar amplicons using 16S rRNA and *KMT1* genes that produced 1 471 and 460 bp products respectively. The molecular identification should be carried out after initial routine diagnostic identification. The results obtained from this study also lead to different cultures of *P. multocida* and this may help to determine those antigens that are responsible for protection within the same group of farm animals. Based on the results of this study, we can recommend that the diagnosis of *P. multocida* strains should not be based on colonial morphology alone, but we must use genetic characterization that the molecular identification methods are decreasing the time required for bacterial identification in the medical laboratories and allow to detection of *P. multocida* strains from clinical samples and we suggested that combined sequences

from 16S rRNA and *KMT1* genes were required to complete identification of *P. multocida* isolates.

In conclusion, based on the molecular study conducted, we found that all *P. multocida* isolates recovered from clinically sick of selected animals gave similar amplicons by using *KMT1* gene and 16S rDNA. Respiratory affections of small ruminant especially pneumonia are regarded as a frequent cause of economic losses in lambs and kids and its etiology involves many different factors including bacteria such as members of family Pasteurellacaea. These bacteria are fastidious organisms and complex in their antigenicity, therefore, the conventional methods for identification are not valuable needing to more recent and discriminating techniques which include various DNA- and RNA-dependent techniques. These results indicated the wide range host of *P. multocida* strains and it can infect different animal species. The 16S rRNA gene sequence showed the role of buffalo as a reservoir for *P. multocida* to cattle independent of virulence genes transmission.

## Conflict of interest statement

We declare that we have no conflict of interest.

## References


[1] Dardeer MA, Hanaa AA, Sahar AO, Hassan AM. Antigenic and genetic variability among mycoplasmas isolated from sheep and goat. *Egypt J Comp Pathol Clin Pathol* 2006; **19**(3): 313-31.

[2] Hotchkiss EJ, Dagleish MP, Willoughby K, McKendrick IJ, Finlayson J, Zadoks RN, et al. Prevalence of *Pasteurella multocida* and other respiratory pathogens in the nasal tract of Scottish calves. *Vet Rec* 2010; **167**: 555-60.

[3] Shayegh J, Sharaf JD, Mikaili P, Namvar H. Pheno- and genotyping of *Pasteurella multocida* isolated from goat in Iran. *Afr J Biotechnol* 2009; **8**: 3707-10.

[4] Rudolph KM, Hunter DL, Foreyt WJ, Cassirer EF, Rimler RB, Ward AC. Sharing of *Pasteurella* spp. between free-ranging bighorn sheep and feral goats. *J Wildl Dis* 2003; **39**: 897-903.

[5] Blackall PJ, Norskov-Lauritsen N. Pasteurellaceae-the view from the diagnostic laboratory. In: Kuhnert P, Christensen H, editors. *Pasteurellaceae: biology, genomics and molecular aspects*. Norfolk: Caister Academic Press; 2008, p. 227-59.

[6] Hunt ML, Adler B, Townsend KM. The molecular biology of *Pasteurella multocida*. *Vet Microbiol* 2000; **72**: 3-25.

[7] Srinivasan R, Karaoz U, Volegova M, MacKichan J, Kato-Maeda M, Miller S, et al. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS One* 2015; **10**: e0117617.

[8] Christensen H, Bisgaard M. The genus *Pasteurella*. *Prokaryotes* 2006; **6**: 1062-90.

[9] Jabbari AR, Esmailzadeh M, Moazeni Jula GR, Moosavi Shoushtari M. Genetic variations of avian *Pasteurella multocida* as demonstrated by 16S-23S rRNA gene sequences comparison. *Iran J Vet Res* 2008; **9**(3): 240-4.

[10] Prabhakar TG, Prabhakar P, Venkadabadi. Molecular characterization of *Pasteurella multocida* isolated from an incidence of sheep pasteurellosis in karamadai hill tract of Tamil Nadu. *Tamilnadu J Vet Anim Sci* 2010; **6**(2): 81-7.

[11] Deressa A, Asfaw Y, Lubke B, Kyule MW, Tefera G, Zessin KH. Molecular detection of *Pasteurella multocida* and *Mannheimia haemolytica* in sheep respiratory infections in Ethiopia. *Int J Appl Res Vet Med* 2010; **8**(2): 101-8.

[12] Güler L, Gündüz K, Sarişahin AS. Capsular typing and antimicrobial susceptibility of *Pasteurella multocida* isolated from different hosts. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi* 2013; **19**: 843-9.

[13] Hakim AS, Bakry MA, Ata NSS, Zaki MS. Role of molecular techniques in characterization of bacteria causing pneumonia in small ruminants. *Life Sci J* 2014; **11**(6): 147-53.

[14] Ozbey GA, Kilic HB, Ertas AM. Random amplified polymorphic DNA (RAPD) analysis of *Pasteurella multocida* and *Mannheimia haemolytica* strains isolated from cattle, sheep and goats. *Vet Med* 2004; **49**: 65-9.

[15] Srivastava S, Singh V, Kumar V, Verma PC, Srivastava R, Basu V, et al. Identification of regulatory elements in 16S rRNA gene of *Acinetobacter* species isolated from water sample. *Bioinformation* 2008; **3**: 173-6.

[16] Hall TA. *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT*. Oxford: Oxford University Press; 1999, p. 95-8.

[17] Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 2004; **5**: 150-63.

[18] Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJ. Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *J Clin Microbiol* 1998; **36**: 1096-100.

[19] Dey S, Singh VP, Kumar AA, Sharma B, Srivastava SK, Singh N. Comparative sequence analysis of 16S rRNA gene of *Pasteurella multocida* serogroup B isolates from different animal species. *Res Vet Sci* 2007; **83**: 1-4.

[20] Shayegh J, Parvizi M, Hejazi MS. Diversity of caprine and ovine *Pasteurella multocida* isolates based on 16S rRNA gene sequencing. *Iran J Vet Res* 2010; **11**: 373-8.

[21] Davies RL. Genetic diversity among *Pasteurella multocida* strains of avian, bovine, ovine and porcine origin from England and Wales by comparative sequence analysis of the 16S rRNA gene. *Microbiology* 2004; **150**: 4199-210.

[22] Balakrishnan G, Roy P. Isolation, identification and antibiogram of *Pasteurella multocida* isolation of avian origin. *Tamilnadu J Vet Anim Sci* 2012; **8**(4): 199-200.

[23] Król J, Bania J, Florek M, Pliszczak-Król A, Staroniewicz Z. Polymerase chain reaction-based identification of clinically relevant Pasteurellaceae isolated from cats and dogs in Poland. *J Vet Diagn Invest* 2011; **23**: 532-7.

[24] Munir R, Shahwar D, Farooq U, Nawaz I, Shahzad I, Khanum A. Outer membrane protein profiling of *Pasteurella multocida*. *Pak Vet J* 2007; **27**: 1-4.

[25] Borowski SM, Silva SC, Schrank I, Cardoso M. Toxin detection in *Pasteurella multocida* strains isolated from swine lungs in the state of Rio Grande Do Sul, Barazil. *Arquivos da Faculdade de Veterinária* 2001; **29**: 79-85.

[26] Liu D, Lawrence ML, Austin FW. Specific PCR identification of *Pasteurella multocida* based on putative transcriptional regulator genes. *J Microbiol Methods* 2004; **58**: 263-7.

[27] Wei B, Cha SY, Kang M, Park IJ, Moon OK, Park CK, et al. Development and application of a multiplex PCR assay for rapid detection of 4 major bacterial pathogens in ducks. *Poult Sci* 2013; **92**(5): 1164-70.