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Molecular Characterization of *Pasterulla Multocida* in Fattening Bovine

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PASTEURELLA multocida infection has serious complications for both human and animal health. In bovines, it is the predominant cause of fatal pneumonia, with a mortality rate up to 100%, especially in severe undiagnosed cases. Therefore, this study was conducted for molecular identification and characterization of *P. multocida* in nasal swabs and pneumonic lung tissue in cattle calves less than one year suffered from pneumonia and respiratory disorders. The detection of *P. multocida* was higher in pneumonic lung tissue (40%) than in nasal swabs. Only 37 out of 109 collected samples were positive for *P. multocida* using bacteriological culture and those samples were confirmed by PCR targeting *Kmt1* gene. The phylogenetic analysis of local *P. multocida* isolate revealed close relation with other *P. multocida* strains from bovine from Egypt, China, USA and Canada. Consequently, establishing a potent epidemiological surveillance program is necessary to decrease the spreading of disease and the economic losses among fattening calves.

Keywords: *P. multocida*, PCR, *Kmt1*gene, phylogenic analysis, Sequencing.

Introduction

Pneumonic pasteurellosis is a widespread clinically important veterinary disease caused by infection with bacteria of the genus *Pasteurella*. The most frequently detected species in this genus, reported as *Pasteurella multocida* (*P. multocida*). Additionally, the presence of *P. multocida* in both commensal and pathogenic forms makes it a significant concern for the global health community [1, 2]

P. multocida is a Gram-negative bacterium characterized by its ability to encapsulate particularly evident in virulent strains, it is also a non-motile, non-endospore-forming, small, and pleomorphic bacterium. On sheep blood agar plates after 24 h of incubation at 37ºC colonies were displayed as smooth, round, greyish or yellowish, glistening, translucent, and lacking hemolytic activity with 1-2 mm in diameter colonies. [3-5].

P. multocida implicated in a wide range of economically significant serious field diseases in domestic animals, including but not limited to haemorrhagic septicaemia and shipping fever pneumonia in cattle and water buffaloes, enzootic pneumonia in sheep and goats, atrophic rhinitis in pigs [2, 3, 6].

Pasteurellosis is a worldwide disease reported in numerous countries in Asia, Africa, the Middle East, and southern Europe. It is endemic in Egypt and is considered one of the highly contagious respiratory diseases that dent cattle husbandry in

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Egypt, as respiratory infections cause catastrophic economic repercussions and fatal complications in vulnerable young calves due to pneumonia [7-9].

Pasteurellosis poses a substantial threat for livestock owing to its high mortality rate and the potential for rapid transmission within and among herds. The disease transmits mainly through inhalation of nasal secretions or exhaled droplets from infected animals [10].

Pasteurellosis is manifested as acute, sub-acute and chronic forms. The typical case is manifested as a high fever of 40 to 41°C, bilateral mucopurulent nasal discharge, mouth frothing, coughing, varying degrees of polypnea and dyspnea, rumen atony, and evidence of bronchopneumonia. Septicaemia is a hallmark feature across all disease forms. In certain cases, lymphadenopathy, particularly those located around the head and neck area is manifested as edematous swelling of submandibular and brisket regions [1, 8, 11].

In recent years, there is a statistically significant upsurge in clinical bovine pneumonic pasteurellosis affecting both unweaned and older calves with a predominance of *P. multocida* as the primary etiological agent, independent of co-infections by other *Pasteurellaceae* bacteria [11].

It›s significant to emphasize that clinical signs of bovine pasteurellosis can overlap with other respiratory diseases in cattle, posing a significant diagnostic challenge. Consequently, a definitive diagnosis usually requires laboratory testing [1].

Pneumonic pasteurellosis is endemic in Egypt. It has been reported in Egypt in many previous studies with different infection rates 12.76%, 34.4%, 18.2% and 50% [12], [13-15]. Additionally, infection rate is high in young unweaned male cattle calves. It has the potential to cause mass mortality events with up to 100% mortality.

Traditionally, the ultimate diagnosis of bacterial infection relies principally on classical bacteriological techniques, by isolation and identification of suspect bacterial colonies through microscopy and biochemical tests. Nonetheless, the advent of modern genotypic characterization can provide dependable techniques for expediting the detection of diverse bacterial species. Furthermore, sequencing-based techniques targeting specific genes are critical tools for the precise identification of infectious bacterial pathogens, furthering our diagnostic capabilities [9, 16].

Recently, PCR, one of the nucleic acid-based assays, consider a powerful tool facilitate the direct detection of *P. multocida* from clinical samples or scant cultured bacterial cells, thereby lead to a significant enhancement the sensitivity and reduction the time required for definitive *P. multocida* identification [17, 18].

Understanding of the genetic diversity and molecular characterization of *P. multocida* are critical for diagnosis, epidemiology investigation, effective vaccine formulation, and facilitate regional monitoring of circulating strain variants [19, 20].

The *kmt1* gene is significant for the molecular detection and characterization of *P. multocida*. The kmt1 gene encodes the outer membrane protein, generating an amplification product specific to all strains of *P. multocida*. Comparative analyses with alternative target genes, including 23S rRNA and transcriptional regulator genes, have revealed superior sensitivities and specificities of the *kmt1* gene. hence, the *kmt1* gene is recommended as an accurate identification marker of *P. multocida* [19, 21].

Therefore, the current study was carried out for identification and molecular characterization of *P. multocida* in young cattle calves suffered from pneumonia in some localities in north of Egypt.

Material and Methods

Ethical statement

This study conducted in adherence to the principles of clinical practices and obtained approval from the Animal Experiment Ethical Committee of the Faculty of Veterinary Medicine, Benha University, under the reference number BUFVTM 24-04-2023.

Animals and sampling

A total of 109 samples (79 deep nasal swabs, 30 pneumonic lung tissues) were collected from some localities in north of Egypt (Gharbia, Minufiya, Qalyubia and Cairo) governorates form cattle calves less than one year. The deep nasal swabs samples were taken from cattle calves showing respiratory symptoms such as (coughing, sneezing, rapid breathing, nasal discharge, and loss of appetite along with a rectal temperature above

39.5°C). Pneumonic lung tissues were collected from El-Basateen abattoirs in Cairo governorate from slaughtered cattle calves) that showed signs of pneumonia in postmortem inspection.

Isolation of P. multocida

The isolation of *P. multocida* was performed onto blood agar media supplemented with 7% defibrinated sheep blood (Oxoid, UK) and incubated aerobically for 24 hr at 37°C and biochemical identification of *P. multocida* from the samples were performed according to methods described by Dousse, Thomann, Brodard, Korczak, Schlatter, Kuhnert, Miserez and Frey [22] and Quinn, Markey, Leonard, Hartigan, Fanning and Fitzpatrick [23].

Molecular detection of P. multocida

The DNA was extracted from bacterial isolates which previously identified as *P. multocida* based on culturing with commercial kit (QIAamp DNA Mini Kit, Qiagen GmbH, Germany) according manufactures instructions. All the extracted DNA were stored at - 20 ◦C until further analysis.

The PCR assay was performed using specific pair of primers forward (ATCCGCTATTTACCCAGTGG) and reverse (GCTGTAAACGAACTCGCCAC) targeting *Kmt1* gene (460bp) for *P. multocida* [5]. All PCR amplifications were performed in 25-μL volume containing 12.5 μl of 2X Taq PCR Master Mix (Qiagen, Germany), 1 μl of each primer (Metabion company, Germany), 5 μl of DNA template and 5.5 μl of PCR grade water (Jena Bioscience, Germany) under the following conditions: initial denaturation step at 94◦C for 5 min, followed by 35 cycles of secondary denaturation at 94◦C for 30 sec, annealing at 55◦C for 40 min, and extension at 72◦C for 1 min. After the last cycle, the mixture was incubated at 72 ◦C for 10 min for final extension step. The amplification products were analyzed by electrophoresis on a 1.5% agarose gel.

Kmt1 gene sequencing and phylogenetic analysis

The PCR product of highest DNA concentration sample was purified using QIAquick PCR product extraction kit (Qiagen, Valencia, CA), followed by sequencing using the same primers of conventional PCR. The sequencing was performed using big dye Terminator V3.1 cycle sequencing kit (PerkinElmer, Foster city, CA) in Applied Biosystems 3130 genetic analyzer (HITACHI, Japan) according to instruction of manufacture. The purified PCR product was sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA) according to instructions of manufacture. The sequence of *Kmt1* gene was aligned with other published sequences in GenBank using the CLUSTAL W multiple sequence alignment program, version 12.1 of MegAlign module of Lasergene DNAStar software Pairwise (Madison, Wisconsin, USA) which was designed by Thompson, Higgins and Gibson [24] and Phylogenetic analyses were constructed using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 [25]

Results

Culture and biochemical result

On blood agar, *P. multocida* was non haemolytic and appeared as moderate size (1-2 mm) in diameter, round, translucent and greyish mucoid colonies. Biochemically, *P. multocida* was oxidase and catalase positive and urease and citrate negative. It was indole positive, also it abled to ferment glucose and sucrose without H_2S or gas production.

Molecular identification of P. multocida

The number of isolates was identified as *P. multocida* using PCR assay from nasal swabs was 25 (31.6%) and 12 (40%) from pneumonic lung tissue, Fig 1.

Kmt1 gene sequencing and phylogenetic analysis

The sequencing for one of highly concentrated DNA for *P. multocida* was performed based on *Kmt1* gene. The sequence was subjected to nucleotide and amino acids alignment analysis and phylogenetic analysis to assess the genetic similarity with local or global strains. *P. multocida* isolate has been submitted to GenBank under accession number OQ828705. Nucleotide and amino acids sequence identities between *P. multocida* isolate in this study and 26 *P. multocida* strains uploaded in gene bank showed (98.4-100%) identity.

The phylogenetic analysis revealed that the sequence of (*P. multocida* PM-GI-AGR-EG23) has a close relation with other strains of *P. multocida* previously reported in Egypt (MZ419557, MZ419556, KR006979), China (CP033599, CP006976), USA (CP008918, AE004439) and Canada (CP045724) in bovine , additionally; has a close relation with *P. multicda* strains reported in animals other than bovine as (MT263081,MT263078) in camels, (CP040848) in pigs and (MH068783) in goats as shown in Fig.2.

Discussion

Pneumonic pasteurellosis is considered one of the substantial economic burdens on the feedlot and dairy industries. Its global impact is devasting, surpassing one billion dollars annually in losses attributed to the disease within the beef cattle sector alone [26].

P. multocida, a highly contagious bacterium, is the etiological agent of haemorrhagic septicaemia, a fatal disease of cattle and buffaloes. Characterized by acute, highly fatal septicaemia with significant morbidity and mortality, which manifests as catastrophic epizootics in numerous African and Asian countries [27].

This study aimed to molecular identification and characterization for *P. multocida* in nasal swabs and lung tissues in young calves suffered from pneumonia in some localities in north of Egypt

Generally, traditional diagnosis of *P. multocida* through culturing method has the advantage of easy to obtain a definitive diagnosis through isolation and identification of a pure culture from the fatal cases on the other hand, it can be difficult to isolate in field screening for carriers.

The prevalence of *P. multocida* exhibit considerable variability and may sometimes be increased and varied depending on the source of isolation [28]. In present study, the overall prevalence of *P. multocida* depending on source of isolation was higher in pneumonic lung tissue than in nasal swabs. This result came in agreement with the previously reported findings from Egypt by El-Jakee, Ali, El-Shafii, Hessain, Al-Arfaj and Mohamed [13] , Bostan, Torky, Ahmed and Hassan [18] and, Algammal, Enany, El-Tarabili, Ghobashy and Helmy [29].

Generally, this results can be elucidated by the ease of isolation of pure culture from lung tissues of fatal cases, contrasting the difficulty isolating in field screening for carriers as The bacteria are not consistently present in the nasal secretions or body fluids of sick animals [1]. Furthermore, once *P. multocida* establishes colonization of the upper respiratory tract, it initiates an inflammatory response in the bovine respiratory tract, leading to *Pasteurella* attacking the lung tissues through repeated aspiration of infected droplets or sloughed tissue. The inflammatory response, coupled with the effect of virulence factors of *P. multocida*, contributes to damage in lung tissues, thereby making it the dominant isolate in pneumonic lung tissue [30, 31]

Due to limitations linked with conventional diagnostic methods for bacteria, molecular methods provide highly specific and efficient tools for promptly detection and confirmation of Pasteurella species [18, 27]. Additionally, it was also assured that PCR amplification conducted directly on bacterial colonies or cultures constitutes an extremely rapid and sensitive approach for identifying *P. multocida* [17].

So, for more confirmation, primers for the *kmt1* gene encoding the outer membrane of *P. multocida* were designed. The results of PCR confirm that bacterial cultures previously identified as positive for *P. multocida* were indeed positive for the *kmt1* gene, aligning with findings by Abbas, Abd El-Moaty, Zaki, El-Sergany, El-Sebay, Fadl and Samy [19] and Abed, El-Seedy, Hassan, Nabih, Khalifa, Salem, Wareth and Menshawy [27] who used *kmt1* gene for identification of *P. multocida* as The *kmt1* gene has shown superior sensitivity and specificity in detecting *P. multocida* producing an amplification product unique to all strains of *P. multocida*. moreover, it has served as a marker gene for *P. multocida* in several PCR methods [19, 21].

The sequence analysis for positive *P. multocida* isolate was applied to assess its genetic homology with different local or global *P. multocida* strains. The sequencing of *P. multocida* isolate from the present study shared high nucleotide and amino acids identity with strains reported from cattle and other animal species in Egypt and other countries. Such findings can be attributed to the fact that animal movement, animal importation and animals are transported between different countries, and hence, bacterial infection can easily be found in various places [9, 32]

In addition, there is a close relationship between *P. multocida* strains of bovine and other strains reported in other animals as pigs, camels and goats which made *kmt1 gene* an important epidemiological tool and help in selecting the antigen responsible for protection within the same group and thereby aid in the development of new *Pasteurella* vaccines [33].

Also, the present results suggest that circulating *P. multocida* strains are genetically conserved with a limited sequence diversity between strains compared to previous reported data.

Conclusion

P. multocida was traditionally isolated from cattle calves and confirmed by PCR technique. *Kmt1* gene was partially sequenced and phylogenetic analysed. Our findings demonstrated that *P. multocida* strain was highly related to several local and global isolates. Moreover, correlated with isolates from animals other than bovine confirming enter-and intra species transmission. Further epidemiological studies are recommended for appropriate control, prevention strategies, more specific and effective vaccines formulation.

Conflicts of interest

The authors declared no competing interests.

Funding statement

Non

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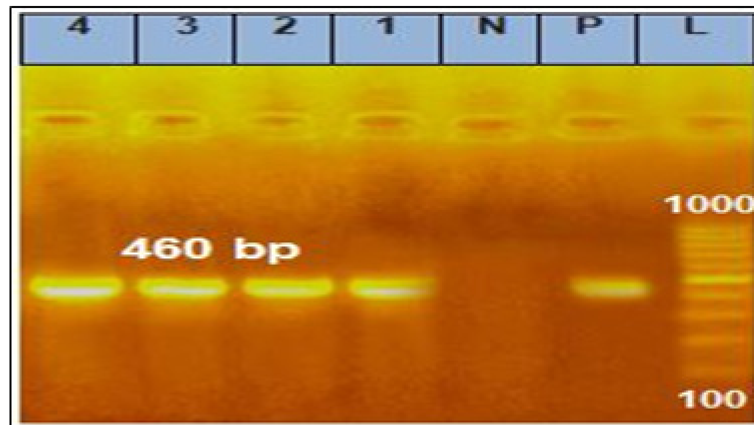

Fig. 1. PCR targeting *Kmt1* gene for detection of *P. multocida* isolates. lane L: ladder (100-1000 bp). P: control positive. N: control negative, Sample 1 and 2 from nasal swabs and samples 3 and 4 from lung tissue samples

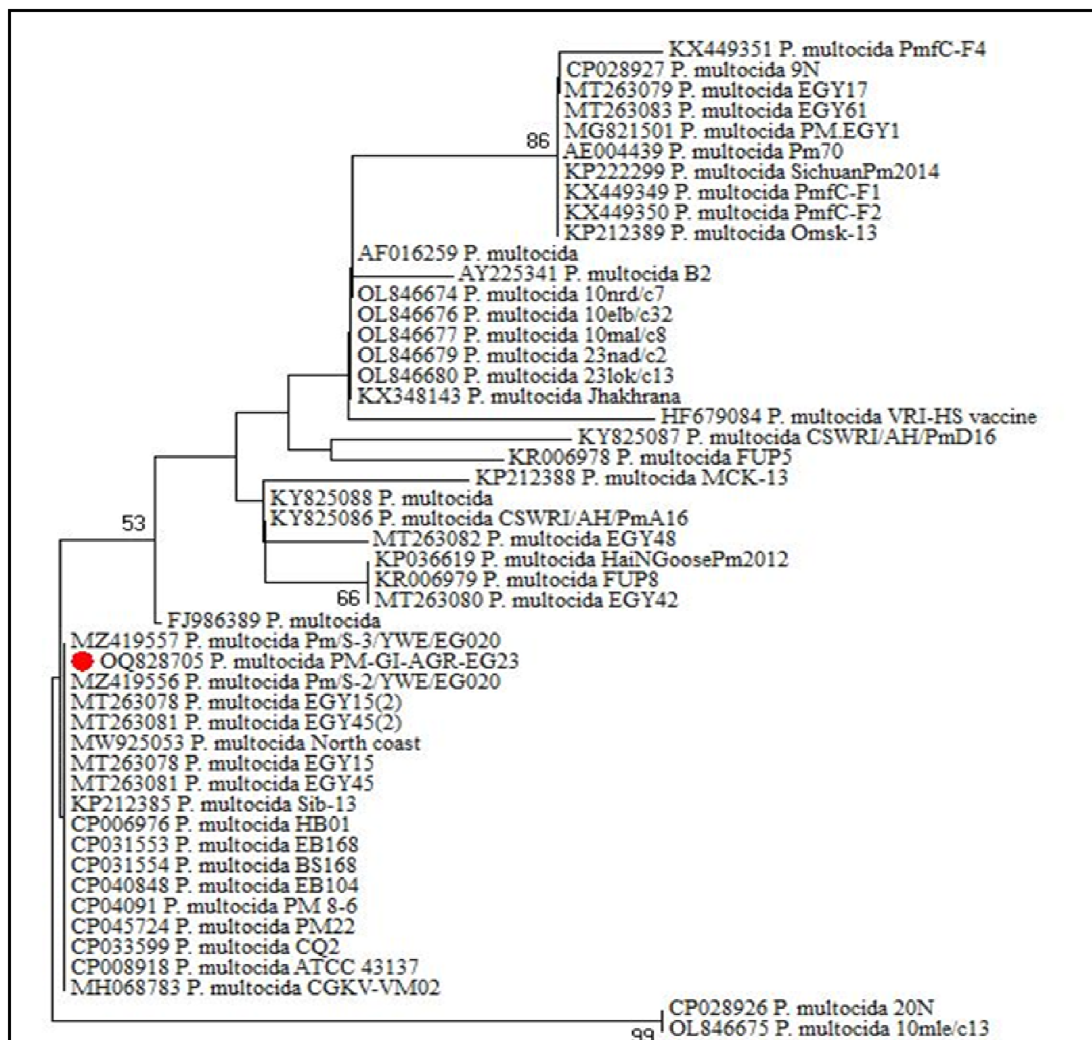

Fig. 2. Phylogenetic tree for KMT1 gene of P. multocida showing the relationship between our isolate (OQ828705) and the closely related members of Pasteurella obtained from NCBI database.

التوصيف الجيني لباستريلا ما لتوسيدا بأبقار التسمين

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إن عدوى الباستريلا مالتوسيدا لها مضاعفات خطيرة على صحة الإنسان والحيوان. بالمجترات، تعد واحد من الأسباب الأساسية للالتهاب الرئوي القاتل، حيث يصل معدل الوفيات إلى ١٠٠٪، خاصة في الحالات الشديدة غير المشخصة. لذلك، أجريت هذه الدراسة للتعرف الجزيئي وتوصيف بكتيريا باستريلا مالتوسيدا في المسحات الأنفية وأنسجة الرئة المصابة بالالتهابات الرئوية في عجول الأبقار الأقل من عام والتي تعاني من الالتهاب الرئوي واضطرابات الجهاز التنفسي. تم تشخيص الباستريلا مالتوسيدا بنسبه اعلي (40٪) في أنسجة الرئة الرئوية عنها في مسحات الأنف. فقط ٣٧ من أصل ١٠٩ عينة تم جمعها كانت إيجابية بالنسبة لباستريلا مالتوسيدا باستخدام العزل البكتريولوجي وتم تأكيد تلك العينات بواسطة تفاعل البلمرة المتسلسل والذي يستهدف جين Kmt1. وكشف التحليل الوراثي لعزلة الباستريلا مالتوسيدا المحلية عن وجود علاقة وثيقة مع سلالات الباستريلا مالتوسيدا الأخرى من الأبقار من مصر والصين والولايات المتحدة الأمريكية وكندا. وبالتالي، فإن إنشاء برنامج فعال للمتابعة الوبائية أمر ضروري لتقليل انتشار المرض والخسائر الاقتصادية بين عجول التسمين.

الكلمات المفتاحية: P. multocida، PCR، Kmt1gene، التحليل التطوري، التسلسل.