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Molecular characterization of *Pasteurella* species isolated from slaughtered cattle in Assiut abattoirs and molecular detection of some antibiotic resistance genes

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ABSTRACT

Pasteurella multocida (*P. multocida*) is one of the most predominant pathogenic bacterial agents causing respiratory diseases in different types of animals with considerable economic losses and unfavorable prognosis in Egypt. Recently, *P. multocida* has exhibited resistance to the most used antibiotics in the veterinary field. So, this study was carried out to investigate the prevalence of both virulence and antibiotic resistance genes among *P. multocida* isolated from apparently healthy and diseased bovine lungs. Only 10 out of 60 lung samples were collected from the different slaughterhouses and just 9 were confirmed finally positive for *P. multocida* by PCR using *Kmt1*. Antibiotic susceptibility testing of the recovered isolates revealed that they were all multidrug/resistant (MDR) with a predominance of resistance to erythromycin (100%) and most of them by (90 and 80 %) for amoxicillin and doxycycline, respectively; followed by 60 and 50 for ceftriaxone, gentamicin. All of the obtained isolates were promoting consistency through PCR screening for a few relevant common antibiotic resistance genes. All MDR *P. multocida* isolates had at least one gene for antibiotic resistance, mostly *AphA1* and *BlaROB1* (100%), and only 60% of them had the *ermX* gene. Antibiotic resistance genotyping showed that the majority of the isolates, (60%) of isolates, having three genes producing identical resistance phenotypes had multiple antibiotic resistance genes. Our results demonstrated that pathogenic *P. multocida* strains carrying virulence and antibiotic resistance genes may originate from cattle. Therefore, it is evident that there is an urgent need for the judicious use of antibiotics in bovine treatment systems to successfully mitigate the propagation of drug resistance across *P. multocida* species.

Introduction

The enigmatic pathogen *Pasteurella multocida* (*P. multocida*) is known for being linked to a variety of respiratory syndromes that can affect a diverse range of host species, including cattle, pigs, domestic and wild birds, rabbits, cats, dogs, and fish (Carrera and Notario, 2023). It is a typical resident of the respiratory tract of healthy animals (Myer and Myers, 2023). One of the major infectious diseases affecting the cattle industry, bovine respiratory disease (BRD) costs fattening operations a large amount of money due to increased mortality, expensive treatment expenses, or slowed growth (Jia *et al*., 2023). One of the bacterial pathogens most frequently implicated in BRD outbreaks during the past few years specially that have received vaccinations against other BRD-related infections is *P. multocida*, all of which confirms the relevance of it in BRD (Bernal *et al*., 2023; Saco and Bassols, 2023). Isolation of this organisms from conventional cultural even by using different specific antibiotic as (amikacin, vancomycin and amphotericin B) not provide accurate diagnosis for *P. multocida* so that, molecular genetic (*Kmt1*) confirmation was recommended. The polysaccharide capsule and lipopolysaccharide (LPS) are of the major important virulence determinants contributed in the pathogenesis of *P. multocida* (Piva *et al*., 2023). However, many other putative virulence factors are related to pathogenicity including adherence and colonization factors, fimbriae, iron regulating and acquisition proteins, exotoxins and extracellular enzymes. Antibiotics have been used widely for the treatment of pasteurellosis in animals, their prolonged and indiscriminate use has led to onset of resistance among various strains (Carrera and Notario, 2023), therefore, limiting therapeutic option. Moreover, the multi-drug resistant (MDR) in pathogenic bacteria from food-producing animals and environmental sources is recognized as a global problem for both veterinary and human medicinal fields (Berman *et al*., 2023). Several studies have proved that the imprudent usage of antibiotics increases the high risk for the selection of resistant bacteria and promotes the spread of resistance genes located on plasmids, integrons, and transposons. Antibiotic resistance of *P. multocida* strains vary according to host origin, time of infection, geographic location, antibacterial pretreatment, and accessibility of the isolates to the resistance genes present in the gene pool (Van Duijkeren *et al*., 2023). But this leads to increased treatment costs, prolonged illness and sometimes death with adverse effects on the economy of Egypt (Yehia *et al*., 2023). Antibiotic susceptibility testing is therefore crucial for the selection of efficient medications by veterinarians in this area. Implementing suitable prophylactic measures will be made easier by information on the antibiotic resistance and virulence traits of various *P. multocida* strains (Berman *et al*., 2023). Antimicrobial resistance can be better understood by analyzing the distribution of resistance genes, especially in Egypt where existing data is quite limited. These knowledge gaps encourage the need for research in theses serious issues. Therefore, the present study aimed to assess the prevalence and pattern of antibiotic resistance of *P. multocida* isolated from lung samples of cattle slaughtered in an industrial abattoir in Assiut city, Egypt.

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Materials and methods

Specimens

After slaughtering, and by using a sterile scalpel, a total of 60 lung samples were collected from different slaughterhouses in the period from January to March 2023, at Microbiology Department, Animal Health Research Institute (AHRI), Assiut branch, Egypt). Samples were transferred in special ice-filled containers. As the organism is sensitive, the samples were delivered to the laboratory not later than 5-6 hours after sampling.

Bacteriological examination

The samples were cultured on blood agar containing amikacin (2 mg/liter), vancomycin (4 mg/liter), and amphotericin B (4 mg/liter); incubated in 37°C for 48 hours. The suspected colonies were subjected to standard biochemical tests as described earlier (Songer and Post, 2005). Then confirmed by PCR using specified gene (Ashrafi *et al*., 2022).

Antibiotic susceptibility

All of *P. multocida* isolates were tested against 9 antimicrobial agents (Oxoid) by the disk diffusion method with the antimicrobial concentration as follows: amoxicillin (10 µg), Ciprofloxacin, ceftriaxone (30), erythromycin (15 µg), gentamicin (10 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole, streptomycin and doxycycline. The tests were carried out according to the guideline of the Clinical and Laboratory Standards Institute (2018). Then, they were incubated at 37°C for 18 h. The inhibition zones were measured with a ruler to the nearest millimeter. The strains were classified as sensitive, intermediate, and resistant, using the zone diameter standards provided by the CLSI (2018).

Detection of virulence and antibiotic resistance genes

The virulence genes and antibiotic resistance genes were tested by the PCR with specific primer pairs listed in Table I. The standard PCR mixture, the thermal cycling procedure and the result analysis step were performed similarly with the capsular typing PCR assays as described above. Except the *tetO*- detecting PCR assays, the extension time of the thermal cycling procedure was increase to 1 min and the assay results were analyzed on 0.8% agarose gel since the expected assay products were 1.8 kb. The appropriate positive and negative controls used in this study were clinical isolates of *P. multocida* that was confirmed in advance by PCR.

Results

Bacteriological examination of samples (n.= 60) showed isolation of *P. multocida* strains in 10% and 23.33% from apparently healthy and diseased lung, respectively with 1.67% total positive from collected samples. That isolated strains were confirmed by using *Kmt1* gene (PCR technique) and proved that nine out of ten were positive for *P. multocida* (Figure 1).

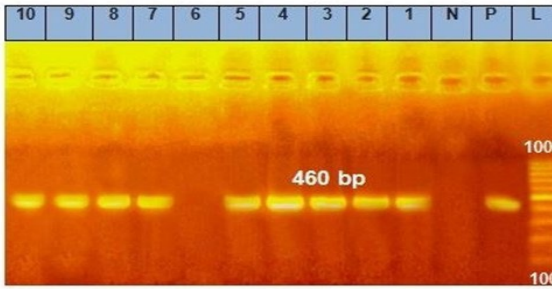

Figure 1. Amplification of *Kmt1* gene (460 bp) specific to *P. multocida*. L: Ladder (Molecular weight marker, 100-1000 bp); Lane P: positive control; Lane N: negative control; Lanes 1-5 and 7-10: positive samples with amplicon size of 460 bp, and Lane 6: negative samples.

Ten strains of *P. multocida* isolated from lung were tested for resistance to 9 antibiotics (Table 2). All the prevalent phenotypes observed were resistant to erythromycin (100%) and most of them were resistant by 90 and 80 % for amoxicillin and doxycycline, respectively; followed by 60, 50, 30 and 20% for ceftriaxone, gentamicin, trimethoprim/sulfa-

Table I. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Kmt1	ATCCGCTATTTACCCAGTGG GCTGTAAACGAACTCGCCAC	460 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	OIE (2012)
AphA1	TTATGCCTCTTCCGACCATC GAGAAAACTCACCGAGGCAG	489 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Klima *et al*. (2014)
BlaROB1	AATAACCCTTGCCCCAATTC TCGCTTATCAGGTGTGCTTG	685 bp	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
ermX	GAGATCGGRCCAGGAAGC GTGTGCACCATCGCCTGA	488 bp	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	

Table 2. Antimicrobial susceptibility testing of 10 *P. multocida* isolates tested by disc diffusion method.

Antimicrobial agents	No. (%) of isolates		
	Susceptibility	Intermediate	Resistance
Amoxicillin	1 (10)	0 (0)	9 (90)
Ciprofloxacin	10 (100)	0 (0)	0 (0)
Ceftriaxone	2 (20)	2 (20)	6 (60)
Erythromycin	0 (0)	0 (0)	10 (100)
Gentamicin	2 (20)	3 (30)	5 (50)
Chloramphenicol	1 (10)	7 (70)	2(20)
Trimethoprim/Sulfamethoxazole	6 (60)	1 (10)	3 (30)
Streptomycin	3 (30)	5 (50)	2 (20)
Doxycycline	1 (10)	1 (10)	8 (80)

methoxazole, chloramphenicol and streptomycin, respectively. While no resistance to ciprofloxacin.

The occurrence of the resistance gene, all tested isolates (100%) were positive for *AphA1*and *BlaROB1*. Where 6 out of 10 positives for *ermX* genes (Figure 2). That may associate to be established between resistance to a given antimicrobial agent and the detection of some of the genes being able to explain the different reaction of susceptibility. Thus, no significant association between the phenotypic and genetic expression because that depend on different variant factors.

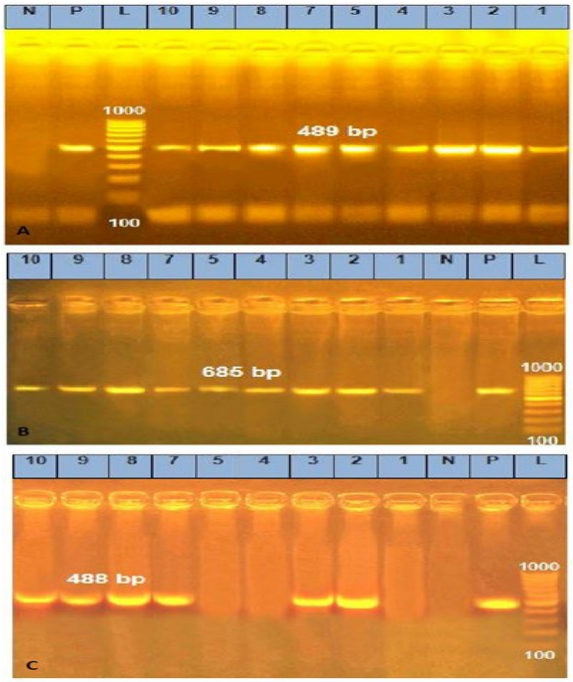

Fig. 2. Polymerase chain reaction assay checking the prevalence of *AphA1* (A), *BlaROB1* (B) and *ermX* (C) genes for *P. multocida* isolates. L: Ladder (Molecular weight marker, 100-1000 bp); Lane P: positive control; Lane N: negative control; Lanes 1-10: samples.

Discussion

Recently, bovine pasteurellosis is the most chronic with high mortality and acute onset endemic disease in majority of nations worldwide. That frequently causes in poor performance, feed conversion and the condition is distinguished by abrupt onset and high mortality. This bovine disease has been on the rise in recent years. With special references to their frequency and pattern of antibiotic resistance is of importance. Based on the obtained results, there is different previous studies with variant distribution and prevalence from region to another according to time of survey. Some of these studies higher than our results by 49.3%, 47.6%, 33.33% for *P. multocida* in some cattle's China, India and England respectively while there is nearly agreement with Ashrafi *et al.* (2022) (11.6%) from normal lung and 21.8% from pneumonic lung tissues (Al-Haj Ali and Al-Balla, 2019). In contrast with the present finding, Haji Hajikolaei *et al.* (2010) recorded 1.6% positive for *P. multocida* in normal lung, Khamesipour *et al.* (2014) isolated 30 from 333 diseased and healthy slaughter cattle which is lower than the presented results. Additional studies recorded all positive (100%) in sheep diseased lung (Kumar *et al.*, 2015); 3 out of 8 clinical cases in India. Sahay *et al.* (2020) recovered *P. multocida* from 374 healthy and infected samples. Piva *et al.*, (2023) could isolate only two *P. multocida* from lung tissue samples. Also, *Kmt1* was also used in the *P. multocida* multiplex capsular PCR typing assay (Boumart *et al.*, 2021). Presumptive isolates of *P. multocida* were confirmed by a PCR assay with primers specific for the amplification of the *Kmt1* gene also all isolates except one gave negative that indicate the most accurate and sensitive of this gene to Egyptian isolates. That indicated the *Kmt1* gene is the most specific gene for determining *P. multocida* in Egypt. In the present study the factors have been detected in *P. multocida* isolated from the lungs of slaughter cattle. The higher frequency of the factors among isolates from pneumonic lungs suggests the role of these factors in disease occurrence. It was pointed out that virulence gene occurrence in *P. multocida* has a strong positive association with the outcome of infection with the organism in cattle (Gatie, 2020). On the other hand, occurrence of the factors in apparently healthy lungs could possibly indicate early infection or contained infection which couldn't lead to disease. It was previously reported that this facultative anaerobic bacterium is commonly found in clinically healthy calves (Berman *et al.*, 2023). Although this genotype is frequently found in *P. multocida* isolates of bovine origin, it has also been frequently linked to other illnesses and hosts, including progressive atrophic rhinitis and pneumonic pasteurellosis in pigs and avian fowl cholera. As a result, it cannot be said to be exclusively linked to cattle or BRD (Bernal *et al.*, 2023).

The antimicrobial results showed that all isolates were resistant to erythromycin with the highest resistance followed to amoxicillin, doxycycline, ceftriaxone and gentamicin that in agreement with Wilson and Ho (2013); Liu *et al.* (2017); Peng *et al.* (2017) and Berman *et al.* (2023). While Michael *et al.* (2012) recorded that *P. multocida* was resistant to a range of antibiotics, including tetracyclines, chloramphenicol, sulphonamides, spectinomycin, streptomycin, enrofloxacin, florfenicol, tilmicosin and tulathromycin. Anholt *et al.* (2017) and Bernal *et al.* (2023) reported that *P. multocida* (55.5%) was resisted to erythromycin; Ashrafi *et al.* (2022) studied that 18.1% of *P. multocida* was resisted to streptomycin. In a similar study, the sensitivity rates of *P. multocida* to enrofloxacin and erythromycin were in line with those of our study (Khamesipour *et al.*, 2014). In contrast, our study all strains sensitive to ciprofloxacin in similar with Myer and Myers (2023). As seen here, the prevalence and antibiotic resistance pattern *P. multocida* significantly varied in different studies. These differences may be due to the changes in climate, season, breeding, commercial practices, treatments, and management strategies of infected cattle (Jia *et al.*, 2023; Aguilar-Vega *et al.*, 2023).

The *AphA1*, *BlaROB1* and *ermX* genes are among the aminoglycoside, β-lactam and macrolides resistance determinants, respectively. They commonly observed in many pathogenic bacteria but there is little data about these genes in *P. multocida* as survey in Egypt. This study demonstrated a high prevalence of positive *AphA1* gene (100%) also all isolates harboring *BlaROB1* gene that indicated the resistance of theses strains to ampicillin and penicillin while only 60% positive for *ermX* gene. These genes were also detected in all 23 *P. multocida* isolates from bovine respiratory infections in a previous study conducted in China (Alhamami *et al.*, 2023). Another recent Canadian study has also detected this gene in most *P. multocida* isolated from nasal swabs of cattle. The current results were higher 0 that detected by Hirsch *et al.*, (2023) and Sheets (2023) but nearly similar with Ambrose *et al.* (2023); Rao *et al.* (2023) and Van Nguyen *et al.* (2023). The majority of the tested genes were not seen in the phenotypic resistant isolates except for the *AphA1* gene, which was found in each of the 10 isolates resistant to aminoglycosides. These findings suggest the possibility of additional antibiotic resistance genes that were not investigated in the current investigation. Last but not least, this investigation showed that the isolates under examination shared virulence and drug resistance genes (Alhamami *et al.*, 2023). These two worrying developments co-occurring proved that virulence has increased in parallel with the development of antibiotic resistance. These findings, where the rise in bacterial virulence coincides with an increase in resistance, were covered in a number of studies (Coque *et al.*, 2023). Essentially, from the theoretical perspective that only occurs when the host shows the disease clinical indications, bacterial virulence is directly associated with the evolution of antibiotic resistance. This means that antibiotic treatment is administered when the virulent bacterial pathogens are present; however, in the absence of bacterial infection, exposure to antibiotics is significantly lower, so the possibility of antibiotic resistance developing as a result of the lack of antibiotic pressure is also becoming lower.

Conclusion

The existence of *P. multocida* in the lung samples obtained from the apparently healthy and diseased cattle was proved. In addition, clear difference was observed between the populations of *P. multocida* recovered from apparently healthy samples versus diseased samples. All the isolates were susceptible to ciprofloxacin, and the highest resistance was observed to erythromycin, amoxicillin and doxycycline. MDR was observed in *P. multocida* species. For the veterinarians, the results highlight the importance of constant monitoring of antibiotic resistance patterns of BRD pathogens to update treatment protocols. Surveillance studies are also necessary to develop strategies for limiting the spread of resistance.

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Conflict of interest

The authors declare that they have no conflict of interest.

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