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Prevalence and antibiotic susceptibility of *Pasteurella multocida* in cattle and buffaloes

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ABSTRACT

Pasteurella multocida (*P. multocida*) infection is considered one of the highly contagious diseases causing pneumonia in bovine with devastating economic setbacks globally. Recently, inappropriate usage of antimicrobial in treatment and control makes *P. multocida* resistance to the most prescribed veterinary antibiotics. The current study aimed to detect *P. multocida* in apparently healthy and diseased (170) cattle and (174) buffalo in four Egyptian governorates, defined some of epidemiological aspect, phenotypic and genotypic detection of antimicrobial resistance of *P. multocida* strains. The overall prevalence in examined cattle and buffalo was 21.2%. The highest infection was in young male (41.5%) in Cairo governorate (24.5%). The antimicrobial susceptibility test of *P. multocida* isolates showed high prevalence of multi-drug resistance to more than one antimicrobial group as high resistance was recorded against Penicillin-G, Ampicillin, oxytetracycline, streptomycin and sulfamethoxazole-trimethoprim but sensitive to cefquinome. The antimicrobial resistant pattern was confirmed by detection of four antimicrobial resistance genes (*tet*H, *erm*X, bla_{ROB-1} and *aph*A1) in four phenotypically drug resistance isolates. The four isolates revealed positive results for resistance genes by PCR assay except one isolate was negative for *erm*X gene. The result confirms the necessity of reliable use of antimicrobials to avoid the development drug resistance and decrease the economic losses in animal production.

Introduction

Respiratory infection poses a significant challenge to the livestock sector, causing catastrophic economic setbacks on a global scale. Various viral and bacterial pathogens contribute to the development of respiratory infections. Among these bacteria, *P. multocida* is frequently detected in calves suffering from respiratory disease and has shown an escalating occurrence in recent outbreaks (Hashem *et al*., 2022; Calderón Bernal *et al*., 2023). The disease is transmitted mainly through inhalation of nasal secretions or exhaled droplets from infected animals. *Pasteurella* infection is considered one of the most economically important diseases as it has the potential to cause mass mortality events with up to 100% mortality also, accounting for approximately 30% of the total cattle deaths worldwide, additionally it may lead to fatal complications especially in young calves which in role increase expenses of control and treatment (Bahr *et al*., 2021; Clemmons *et al*., 2021).

P. multocida commonly resides in the upper respiratory tract and oropharynx of utmost animals as part of their normal flora. However, it has the ability to become pathogenic when the host is under stressful conditions (Kalhoro *et al*., 2015). It causes Pneumonic pasteurellosis and haemorrhagic septicemia in cattle and water buffaloes (Shivachandra *et al*., 2011).

Pneumonic pasteurellosis and haemorrhagic septicaemia are endemic in Egypt and manifested as acute, sub-acute and chronic forms. Acute disease is characterised by fever, edematous submandibular and brisket swelling, respiratory distress, and profuse mucopurulent or bloody nasal discharge, or in the case of an outbreak, conditions characterised by sudden death within 24 hr of onset, while subacute forms of disease are frequently associated with oedema and longer and chronic courses may involve rapid, painful breathing and nasal discharge. Carrier states are also possible (Clemmons *et al*., 2021).

Several risk factors as age, sex and environmental conditions can contribute to the progress and severity of pasteurellosis in bovines. The disease occurs most commonly in young growing male cattle from 6 months to 2 years of age, but all age and sex groups are susceptible. Cold and rainy weather are commonly followed by epidemics of the disease in cattle (Kabeta *et al*., 2015).

Laboratory confirmation can be achieved by isolating and identifying bacteria in a pure culture. Although it is usually straightforward to isolate pure culture from clinically diseased cases, it can pose challenges during field screening for carriers due to the inconsistent presence of bacteria in the nasal secretions or body fluids of sick animals (OIE Manual, 2021).

Generally, infection with *P. multocida* is controlled by broad spectrum antibiotic but compromises treatment and increase incidence of morbidity and mortality has been dramatically increased in recent years. This phenomenon mainly due to emergence of multidrug resistance (MDR) strains of *P. multocida* that led to overturn the effectiveness of even the most powerful antibiotics (Quinn *et al*., 2011; Elalamy *et al*., 2020).

Additionally, in the developing countries there are no guidelines for using antibiotic for treatment and control of bacterial diseases leading to direful problem of emerging and dissemination of antimicrobial resistance (AMR) strains of bacteria and their AMR genes. These resistance genes may by carried on plasmids and transposons or may be on chromosomal genes conferring away for inter-species and intergenic spread of these genes (San Millan *et al*., 2009; Katsuda *et al*., 2012).

In Egypt, studies have confirmed the existence of resistance genes in different bacterial species isolated from various animal sources. For instance, MDR Enterobacteriaceae isolates obtained from diarrheic calves carry AMR genes such as bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$ and class 1 integrons (Meshref *et al*., 2021), also Ahmed and Shimamoto (2011) documented that

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30.4% of Gram-negative bacteria isolated from bovine mastitis cases in some dairy farms in Egypt had at least one antimicrobial resistance gene as the β-lactamase encoding genes, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA}. In the same context, Abdel Aziz *et al*. (2018) in Beni-Suef governorate reported MDR Salmonella spp. in calves' feces had resistance genes for β-lactams, aminoglycosides and macrolides. These results emphasizing the presence of antimicrobial resistance genes in different animals in Egypt, highlighting on the needs for implementation of suitable antibiotic utilization guidelines and effective surveillance measures.

The identification of AMR involves employing culture-based and molecular-based techniques. Culture-based methods depend on detecting phenotypic resistance by assessing bacterial growth in the presence of antibiotics. On the other hand, PCR stands out as the predominant molecular-based approach for AMR gene detection, providing advantages over phenotypic assays, including faster results, greater sensitivity, multiplex targeting capabilities, and a more accurate characterization and identification of antimicrobial-resistant genes (Sundsfjord *et al*., 2004; Fawzy *et al*., 2017; Kaprou *et al*., 2021).

So, mandatory monitoring of abusive usage of antibiotics and testing for antibiotic sensitivity of *P. multocida* isolates are an important clinical concern to manage clinical cases of pasteurellosis (Michael *et al*., 2018).

This study aimed to isolate and identify *P. multocida* recovered from diseased, apparently healthy, and slaughtered cattle and buffaloes' calves in some governorates in north of Egypt. Assessment of some risk factors with prevalence of *P. multocida*. Phenotypic detection of antibiotic resistance in detected *P. multocida* isolates, and molecular detection of some antibiotic resistance genes in some MDR isolates of *P. multocida*.

Materials and methods

Ethical approval

This study followed the principles of good clinical practice and was approved by the Animal Experiment Ethical Committee of Faculty of Veterinary Medicine, Benha University (Approval no. BUFVTM 24-04-2023).

Animals and sampling

In order to determine an appropriate sample size for *P. multocida* in the governorates under study, the Thrusfield formula was used (n=((Z)^2 P(1-P))/d^2) (Thrusfield, 2018). This formula considers the expected prevalence (P) was 18.2% as reported by El-Seedy *et al*. (2020), required precision (d) of 5%, and a confidence level of 95% (Z=1.96). the calculated sample size was 229 and increased to 344 to increase the precision level of the study.

A total of 344 samples include 250 deep nasal swabs from living animals (79 and 71 from diseased cattle and buffalo and 100 from apparently healthy animals), 56 pneumonic lung tissues (30 cattle and 26 buffaloes), 38 bronchial lymph nodes from (11 cattle and 27 buffaloes) were collected from private farms in Gharbia and Minufiya governorates and during veterinary convoys in different villages and towns of Qalyubia Governorate and slaughterhouses from El-Basateen abattoirs near Cairo governorate during the period from September 2022 to July 2023.

The deep nasal swabs samples were taken from cattle and buffalo calves showing respiratory symptoms (coughing, sneezing, rapid breathing, nasal discharge, and loss of appetite along with a rectal temperature above 39.5°C). Samples were also taken from apparent healthy animals.

Culturing of P. multocida

All samples were aseptically collected and placed into individual tubes containing Amies transport medium (Oxoid, UK) and transferred as soon as possible to the laboratory on ice bags in cooling container for bacteriological examination.

Upon arrival at the laboratory, isolation of *P. multocida* was performed according to Quinn *et al*. (2011), the samples were removed from the transport media and placed individually into brain heart infusion (BHI) broth (Oxoid, UK) and incubated aerobically at 37°C for 6-8 h.

A loopful from the broth was streaked onto blood agar media supplemented with 7% defibrinated sheep blood (Oxoid, UK) and incubated aerobically for 24 h at 37°C. The recovered isolates of *P. multocida* were phenotypically and microscopically examined after staining with Gram's stains.

Biochemical identification of recovered P. multocida isolates

Biochemical identification of detected isolates were done according to Quinn *et al*. (2011). Recovered isolates were tested for catalase, oxidase, citrate utilization, indole, urease, sugar fermentation (glucose- sucrose).

Prevalence of P. multocida

The prevalence of *P. multocida* in cattle and buffalo calves was determined in four Egyptian governorates (Kalyubia, Gharbia, Minufiya, and Cairo). Moreover, the relation between sex (male and female), age (younger and older than one year) and *P. multocida* infection was assessed.

Antimicrobial resistance testing

Antimicrobial resistance test was conducted on *P. multocida* isolates using the Kirby Bauer disk diffusion method (Bauer *et al*., 1966) the isolates were tested against ten antimicrobials on Muller Hinton agar. The interpretation was carried out according to (CLSI, 2006; NCCLS, 2002).

Molecular detection of antibiotic resistance genes

The DNA was extracted from *P. multocida* isolates with the QIAamp DNA Mini Kit (Qiagen GmbH, Germany). All the DNA extracts were stored at - 20°C until use. The four selected isolates were previously phenotypic resistance to one or more antibiotic were examined by PCR technique.

The PCR assay was applied to determine the *tet*H, bla_{ROB-1}, *erm*X and *Aph*A1, genes in four *P. multocida* isolates. Primers sequences and am-

Table 1. Primers sequences and amplified products for the targeted genes.

Target gene	Primers sequences	Amplified segment (bp)	Reference
*tet*H	ATACTGCTGATCACCGT TCCCAATAAGCGACGCT	1076	Klima *et al.* (2014)
bla_{ROB1}	AATAACCCTTGCCCCAATTC TCGCTTATCAGGTGTGCTTG	685	
*erm*X	GAGATCGGRCCAGGAAGC GTGTGCACCATCGCCTGA	488	
*Aph*A1	TTATGCCTCTTCCGACCATC GAGAAAACTCACCGAGGCAG	489	

plified products for the targeted genes are illustrated in Table, 1. All PCR amplifications were performed in 25 µL volumes 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). The temperature and time conditions of the primers during PCR are shown in Table 2. The amplification products were analysed by electrophoresis on a 1.5% agarose gel.

Statistical analysis

All data statistically analysed with SPSSV17 using Student's t-test (Geisser-Greenhouse's epsilon) with $p < 0.05$ and 95% confidence interval. Statistical analyses were performed for studying the difference between two values normally distributed.

Results

Prevalence of P. multocida

The overall prevalence of *P. multocida* in cattle and buffalo calves in areas under study was 21.2% (73/344), the higher prevalence was observed among cattle calves 27.6% (47/170), while it was 14.9% (26/174) in buffalo calve (Table 3).

Culture character and biochemical identification of P. multocida

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

Risk factors related P. multocida infection

The prevalence of *P. multocida* in cattle and buffalo was varied significantly ($P<0.05$) within different localities under the study. The prevalence was higher among cattle and buffaloes raised in Cairo, it was 41.5% and 24.5%, respectively.

Furthermore, the prevalence of *P. multocida*, increased significantly in young cattle (33.3%) and buffaloes (16.5%) particularly in males (Table 3).

Antimicrobial susceptibility

Kirby Bauer disk diffusion testing of *P. multocida* against antimicrobials frequently used in filed show high frequency of multi-resistance isolates as demonstrated in Table 4.

The *P. multocida* strains isolated from the examined animals exhibited high resistance to Penicillin-G, Ampicillin, oxytetracycline, streptomycin and sulfamethoxazole-trimethoprim but sensitive to cefquinome.

Molecular detection of antibiotic resistance genes

The PCR assay for four resistance gene was performed for four selected *P. multocida* isolates. The results showed detectable band with resistance genes (*tet*H, *erm*X bla_{ROB-1} and *aph*A1) in three examined isolates, while one isolates was negative for *erm*X gene (Figures 1 and 2).

Table 2. Cycling conditions for the different primers.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
*tet*H	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 1 min.	35	72˚C 10 min.
*erm*X	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.
bla_{ROB-1}	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
*Aph*A1	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.

Table 3. Prevalence of P. multocida in examined animals in relation to sex age, and location.

Variable	Cattle				Buffaloe			
	Total No of examined animals	No of positive (%)	95% confidence interval	P value	Total No of examined animals	No of positive (%)	95% confidence interval	P value
Location								
Qalyubia	39	9 (23.1)	12.65-38.34		47	3(6.4)	2.19-17.16	
Gharbia	35	3 (8.6)	2.96-22.38	0.044*	22	2 (9.1)	2.53-27.81	0.043*
Menofia	55	18 (32.7)	21.82-45.9		52	8 (15.4)	8-27.52	
Cairo	41	17 (41.5%)	27.75-56.63		53	13 (24.5)	14.93-37.57	
Age								
<1 year	87	29 (33.3)	24.32-43.75	0.015*	127	21 (16.5)	11.08-23.96	0.035*
>1 year	83	18 (21.7)	14.18-31.7		47	5 (10.6)	4.63-22.6	
Sex								
Male	109	33 (30.3)	22.45-39.46	0.024*	98	19 (19.4)	12.78-28.31	0.028*
Female	61	14 (22.9)	14.19-34.91		76	7 (9.2)	4.53-17.81	
Total	170	47 (27.6)	21.48-34.81		174	26 (14.9)	10.4-20.99	

The results are significant if P value < 0.05.

Table 4. Antimicrobial resistance test results against isolated P. multocida.

Antimicrobial	Antimicrobial resistance		
	Sensitive	Intermediate	Resistant
Penicillin-G	-	-	73 (100%)
Ampicillin	-	-	73 (100%)
Cefquinome	68(93.2%)	2(2.7%)	3 (4.1%)
Kanamycin	33 (45.2%)	9(12.3%)	31 (42.5%)
Gentamicin	17 (23.3%)	7 (9.6%)	49 (67.1%)
Streptomycin	-	3 (4.1%)	70 (95.9%)
Oxytetracycline	-	2(2.7%)	71(97.3%)
Chloramphenicol	22 (30.1%)	6 (8.2 %)	45 (61.7%)
Erythromycin	35 (48 %)	14 (19.2%)	24 (32.8%)
Sulphamethoxazol-Trimethoprim	4 (5.5 %)	2 (2.7%)	67 (91.8%)

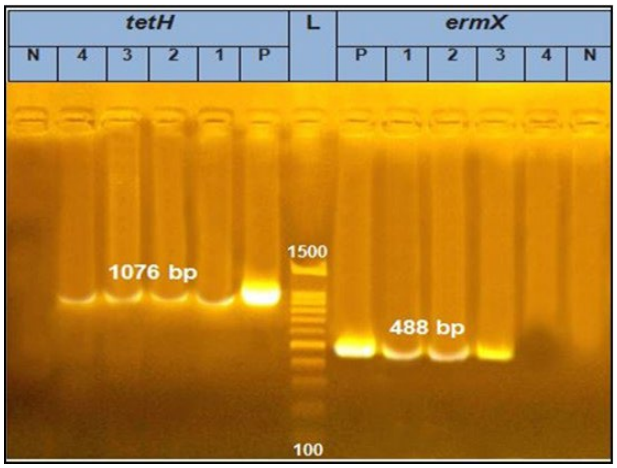

Fig. 1. PCR assay targeting tetH and ermX genes for detection of P. multocida isolates. lane L: ladder (100-1500 bp); P: control positive; N: control negative (1-4) samples

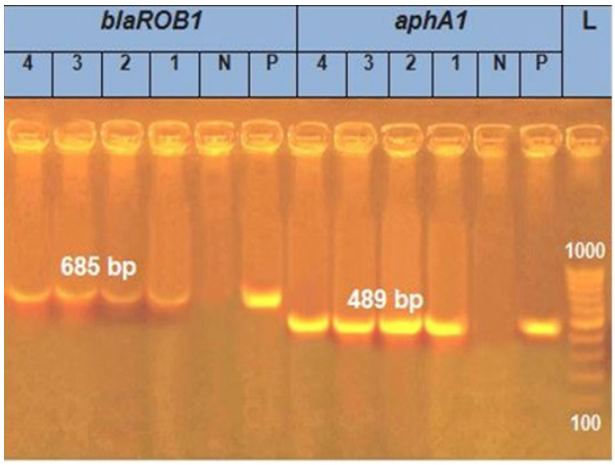

Fig. 2. PCR assay targeting blaROB1 and aphA1 genes for detection of P. multocida isolates. lane L: ladder (100-1000 bp); P: control positive; N: control negative (1-4) samples.

Discussion

Pasteurellosis is one of most prevalent respiratory diseases around the world caused by *Pasteurella* genus. The most commonly reported species in that genus, is *P. multocida* (Hussein, 2021). *P. multocida* is one of the bacterial pathogens most frequently detected in calves affected by respiratory disease where *P. multocida* is commensal in upper respiratory tract of animals, and it has been reported with an increased incidence in the past few years in respiratory diseases outbreaks in Egypt (Abed *et al*., 2020; Calderón Bernal *et al*., 2023).

In our study, the overall prevalence of *P. multocida* was 21.2%. This result is in close vicinity to the previously reported in Egypt by El-Seedy *et al*. (2020) who detected *P. multocida* in bovine by rate 18.2% and similar to findings of Al-Maary *et al*. (2017) in Kingdom of Saudi Arabia, who found that prevalence of *P. multocida* was 26.2%.

Besides, our results were higher than the prevalence rate previously reported by Zaher *et al*. (2014), it was 12.76% in Egypt, in Iran was 9% as reported by Khamesipour *et al*. (2014). These findings might be attributed to the lack of effective vaccinations programs, late detection of diseased condition and lack of effective antimicrobial treatment due to increased AMR.

On the other hand, higher prevalence was reported in Egypt by El-Jakee *et al*. (2016) and Elsayed *et al*. (2021) that was 34.4% and 50%, respectively. In the same context, higher prevalence was reported in North Iran by Jamali *et al*. (2014), it was 83.4%.

Concerning the sex of infected cattle and buffalo, the infection rate of *P. multocida* in this study was higher among males than females. Our findings were in agreement with the findings of Gharibi *et al*. (2017) and Bahr *et al*. (2021). This may be due to male usually reared for meat production that make them usually under stress of transportation form production yard to fattening yard, stress of harsh weather and lack of food and water during transportation. Also collecting animals from different markets, in addition to castration before entry into the feedlot. all of these reasons may contribute to activation of *P. multocida*, which become more pathogenic (Snowder *et al*., 2006; Kabeta *et al*., 2015; Bahr *et al*., 2021)

Concerning the age, cattle and buffalo calves younger than 1 year were found to be more susceptible to take and manifest sings of infection. Bahr *et al*. (2021) reported similar results. This could be explained by young calves have an ill- developed immune system, which makes them more vulnerable to infections. Also, calves are often exposed to stress factors such as transportation, weaning, and mixing with new animals, which can weaken their immune system and make them more prone to infections. In addition, calves are often housed in close proximity to one another, which can increase the potential for the transmission of *P. multocida* (Dabo *et al*., 2008; Shivachandra *et al*., 2011).

Concerning distribution of infection in the areas under study, there was a significant difference between different governorates under study, and it was found that Cairo governorate was the highest in cattle and buffalo, but Gharbia and Qalyubia governorates were the lowest for cattle and buffalo respectively. We speculate that high rate of infection in Cairo governorate, as all samples were collected form slaughtered animals that showed postmortem lesions of *P. multocida* infection. In the same context, the difference between governorates under study may be due to various management and hygienic conditions.

It is indicated that early diagnosis and treatment are very necessary as treatment is of low value once signs of pasteurellosis appear (Shivachandra *et al*., 2011). Even though *P. multocida* is generally vulnerable to a vast range of antimicrobials categories, the occurrence of MDR *P. multocida* has been rising progressively in recent decades. This phenomenon is likely related to the inappropriate utilization of antimicrobials (Petrocchi-Rilo *et al*., 2020).

The antibiotic sensitivity testing of *P. multocida* isolates showed resistance to penicillin-G and ampicillin (100%) and oxytetracycline (97.3%). Meanwhile, showed susceptibility to cefquinome (93.2%) and erythromycin (48%). Our results come in agreement with El-Seedy *et al*. (2020) who report high resistance to ampicillin and oxytetracycline and sensitivity to cefquinome.

Also, our data agree with Elsayed *et al*. (2021) who report that there are high resistance against oxytetracycline (98.2%) but low resistance against β lactam antibiotics (18.2%), in contrary, Cuevas *et al*. (2020) reported that there were high sensitivity to oxytetracycline(100%) and high resistance to penicillin (72.2%).

High resistance against penicillin-G, ampicillin and oxytetracycline in our study may be attributed to haphazard and continuous use of antibiotics without prior drug susceptibility testing or colonization of respiratory system by virulent resistant strains (Nigam, 2015). Additionally, the abusive usage of tetracycline for both the therapeutic and the metaphylactic purposes of *P. multocida* infection, resulting in high percentages of tetracycline-resistant strains (Babetsa *et al*., 2012).

Concurrently with the phenotypic antibiotic resistance assessment, we also explored the existence of specific resistance genes associated with Aminoglycosides, Macrolides, Tetracyclines, β-lactams, which are considered critically significant antimicrobials to veterinary medicine as classified by the World Organization for Animal Health (Góchez *et al*., 2019).

To best of our knowledge, the current study appeared to be the first to report antibiotic resistance genes among *P. multocida* of cattle and buffaloe isolates in Egypt and to confirm the phenotypic resistance profiles of recovered isolates of *P. multocida*.

Four isolates of *P. multocida* with multiple phenotypic resistance results were tested for the presence of antibiotic resistance genes. We explored the presence of resistance genes for Aminoglycosides (*aph*A1 gene), Macrolides (*erm*X gene), Tetracycline (*tet*H gene), β-lactams ($bla_{R\text{-}OB\text{-}1}$ gene)

The results showed that all the examined isolates carried *aph*A1 gene that explain resistance to streptomycin, gentamicin, and kanamycin and this result agreed with Wang *et al*. (2017) that detect *Aph*A1 gene in 100%

of isolates.

Also, four tested isolates carried *tet*H gene that come along with Elalamy *et al*. (2020). Wide spread of *tet*H gene that confer resistance to tetracycline as it is considered to be indigenous for the genus *Pasteurella* and tetracycline is the most commonly used antimicrobial agents in veterinary medicine (Babetsa *et al*., 2012; Góchez *et al*., 2019).

In the same context, the presence of bla_{ROB-1} gene highly confirm resistance of *P. multocida* to Penicillin-G and Ampicillin as shown in Table 4. these results came along with San Millan *et al*. (2009) who detected bla_{ROB-1} gene in all examined strains of *P. multocida* in contrary to Elalamy *et al*. (2020) who recorded it in 8.3% only of isolated *P. multocida* strains . The obtained results confirmed the resistance to the β-lactams especially in Gram-negative organisms due to indiscriminate use of antibiotics in animals medicine (Bush and Bradford, 2016) and presence of bla_{ROB-1} gene which indicated the presence of β-lactamase enzyme that plays a key role in Penicillin-G and Ampicillin resistance strains, in addition to, ROB-1 is the most frequent enzyme conferring β-lactam resistance (San Millan *et al*., 2009).

The *erm*X gene was detected in 3 isolates of *P. multocida* and this gene plays an important role in macrolide resistance. Our result somewhat was in agreement with Awad and El-Hamid (2019), who detected *erm*X gene in 40% of *P. multocida* isolates, but contradict that reported by Elalamy *et al*. (2020) as they did not detect *erm*X gene in their isolates of *P. multocida*.

Resistance toward macrolides antibiotic increased in recent years as these groups of antibiotics are commonly used to control respiratory tract infections in ruminants. Unfortunately, uncontrolled application led to a decline in effectiveness of most of their members as tilmicosin, erythromycin and tulathromycin against *P. multocida*. Resistance may occur by acquired resistance genes (Ujvári and Magyar, 2022).

Conclusion

Global studies highlighted that there is an explosive increase in bacterial resistance, especially MDR. Also, presence of different antibiotic resistance genes in *P. multocida* is a clinically significant issue, as these genes can be transmitted to other bacteria through horizontal gene transfer which may cause severe fatal infections.

Conflict of interest

There are no conflicts of interest declared by the authors.

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