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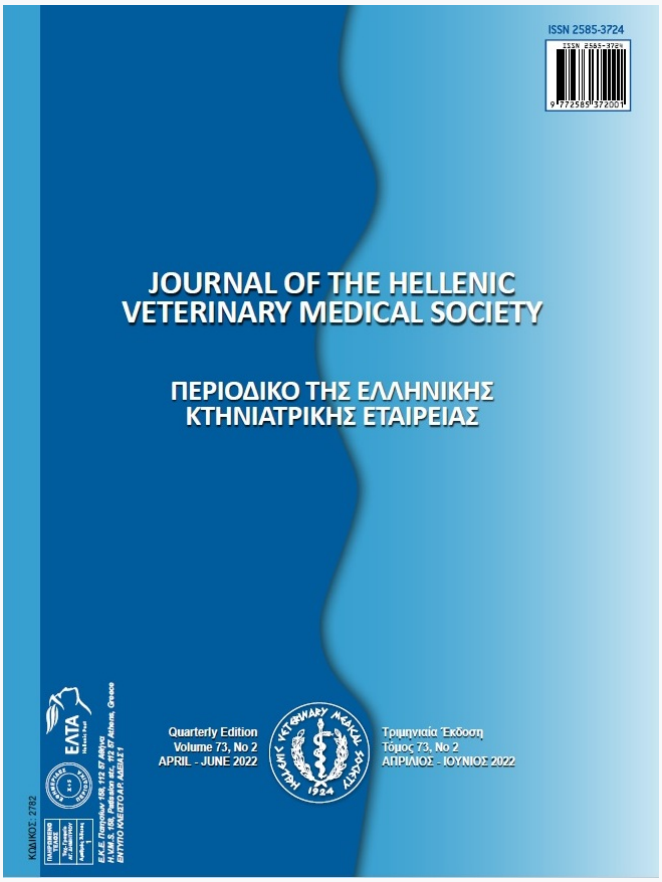

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Characterization of Antimicrobial Resistance Genes of *Pasteurella multocida* Isolated from Chickens in Egypt

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ABSTRACT: In this study, the prevalence of *Pasteurella multocida* in diseased chickens, capsular genotyping, antimicrobial resistance patterns and resistance genes *tetH*, $bla_{ROB-1,}$ *aphA1*, Sul1 and *dfrA* were determined. Lungs, liver and spleen samples were collected from 250 diseased chickens from layers and broiler flocks from El-Gharbia and Kafr El-Sheikh governorates in Egypt for isolation of *P. multocida* in the period from June 2018 to December 2019. Confirmatory identification was done by using PCR for capsular type A antigen. *P. multocida* was isolated from 3.6 % of the diseased chicken. Six isolates of *P. multocida* that examined for detection of capsular type A showed positive results. Antimicrobial resistance patterns were evaluated for all isolates against twenty antimicrobial agents and the results showed 100% resistance totrimethoprim/sulfamethazole, oxacillin and nitrofurantion. Also, strains expressed highly resistant to penicillin, chloramphenicol, rifampicin and ampicillin \sulbactam, while they were sensitive to norfloxacin, clindamycin, cephalexin and cefotaxime. The antimicrobial resistance genes were detected by using PCR and the results showed that all isolates harbored β-lactam-resistant gene bla_{ROB-1} (100%), followed by sulfonamide resistant gene sul1 (50%), tetracycline-resistant gene *tetH* (33.3%) and trimethoprim-resistant dihydrofolate reductase *dfrA* (16.6%).

Key words: Antimicrobial Resistance (AMR) gene*, Pasteurella multocida*, serotype A

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INTRODUCTION

Pasteurella multocida is a Gram-negative bacterium which infects a wide range of animal species, inflicting ailments such as fowl cholera in poultry (Glisson et al., 2003). Fowl Cholera is a serious tremendously contagious disease. It is enzootic disorder and can spread without symptoms inside species. All bird species are affected with fowl cholera globally (Aravinth et al., 2016). Fowl cholera which is typically triggered through serotypes A:1, A:3 or A:4, is a severe systemic disease which happens in domestic and wild birds and outcomes in severe economic losses to poultry industries worldwide mainly fowl and ducks. Different diagnostic techniques have been used for identification of *P. multocida* with variable results. Morphological and biochemical characters for phenotypic characterization are strenuous and tedious. Therefore, molecular assays are most important as they surpass the hinders of phenotyping and furthermore affords information related to the capsular form of *P. multocida* (Rajeev et al., 2011).

Regardless of using antibiotics as an effective tool for controlling *P. multocida* infection, a direful increase in multidrug-resistant (MDR) of *P. multocida* strains is appeared due to excessive utilization of antimicrobials that impose extensive selective pressure on antimicrobial resistance (AMR) genes. This represents a sever challenge for antibiotic use in disease treatment (Khamesipour et al., 2014; Oh et al., 2018). Also the infection caused by MDR bacteria represents costly animal health as a prolonged illness problem (Kilma et al., 2011). This made us requiring new types of antimicrobial drug, but drug discovery and development is complex, expensive, time consuming process involving pharmaceutical manufactures and clinical and academic researches (Projan, 2003). Anti Microbial Resistance (AMR) has found in human, animal, food strains as well as in the environment and can spread between humans, animal and from person to person (Hay et al., 2018).

To date, scant literatures are accessible on MDR avian *P. multocida* isolates as properly as its prevalence amongst fowl flocks in Egypt. Along these lines, the present study determines the incidence of *P. multocida* in diseased chickens in Egypt, capsular typing, AMR patterns and some AMR genes.

MATERIAL AND METHODS

Collection of samples

A total of 750 samples (lungs, spleen and liver) have been collected from 250 diseased and recently dead chickens.The samples correspond to a record of respiratory distress from 175 layer and 75 broiler chickens from the period of June 2018 to December 2019 from four layer and broiler flocks in El-Gharbia and Kafr El-Sheikh Governorates in Egypt. Samples had been packaged in labeled polyethylene bags and transferred to the laboratory for bacteriological examination (Quinn et al., 1994).

Isolation and identification of P. multocida

A loopful from the lungs and spleen and liver had been inoculated on 7% sheep blood agar and incubated for 24-48 h at 37°C (Christensen and Bisgaard, 2010). Suspected *P. multocida* colonies were identified according to the colonial morphology attribute bipolarity and biochemical tests (Glisson et al., 2008) and MacConkey agar media was used for differentiation of *P.multocida* from *Pasteurellaceae* members. Confirmatory identification to isolates by GN 24 according to **(**www.diagnostic.sk**),**isolates were maintained in brain heart infusion (BHI) broth with 25% glycerol at -80°C for further analysis.

Antimicrobial sensitivity testing

Antimicrobial sensitivity testing for *P. multocida* isolates was performed using the disk diffusion method (CLSI, 2016). Antibiotic discs and their concentration (µg/ml) used in this study are shown in table (1).

Molecular identification of capsular type A

Extraction of DNA was performed with The QIAamp DNA Mini Kit (Qiagen, Germany, catalogue no. 51304) according to the manufacturer's instructions. PCR reactions were carried out according to methods of OIE in a final volume of 25µl using the Emerald Amp GT PCR Master Mix (Takara, BIO INC., Japan, code No. RR310A) and OIE primers (2012) for *hya*D-*hya*C gene table (2). The protocol included an initial denaturation step for 5 min at 94°C, followed by 35 cycles of three steps: denaturation at 94°C for 30 sec, annealing of the primers at 55°C for 40 sec and extension at 72°C for 1min. The final elongation takes place at 72°C for 10 min. The mixture also contained a vivid green dye that separated dye fronts into blue and yellow when run on an agarose gel. After PCR, the reaction mixture was applied directly to a gel for analysis.

Table 1. Used antibiotic discs (µg/ml)

Antimicrobial agent	Disc concentration	Antimicrobial agent	Disc concentration
Penicillins - Beta-lactams		*Cephalosporins - Beta-lactams*	
Ampicillin - Sulbactam	20 µg	Cefoperazone	75 µg
Penicillin	10 IU	Cephalexin	30 µg
Ampicillin	10 µg	Cefotaxime	30 µg
Oxacillin	1 µg	*Quinolones*	
Aminoglycosides		Norfloxacin	10 µg
Amikacin	30 µg		
Kanamycin	30 µg	*Folate pathway inhibitors*	
streptomycin	30 µg	Sulfamethoxazole/ trimethoprim	25 µg
Tobramycine		*Macrolides and lincosamides*	
Oxytetracycline	30 µg	Clindamycin	2 µg
Miscellaneous antibiotics		Erythromycin	15 µg
Chloramphenicol	30 µg	*Rifamycins*	
Nitrofurantoin	300 µg	Rifampicin	5 µg
Poly myxin B	300 µg		

Table 2. Oligonucleotide primers sequences

Target gene	Primers sequences (5-3')	Amplified segment (bp)	Reference
***hya*D-*hya*C**	F: TGC-CAA-AAT-CGC-AGT-GAG	**1044**	**OIE. 2012**
	R: TTG-CCA-TCA-TTG-TCA-GTG		
tetH	F:ATACTGCTGATCACCGT	1076 bp	**Klima *et al*., 2014**
	R: TCCCAATAAGCGACGCT		
***bla*$_{ROB-1}$**	F: AATAACCCTTGCCCCAATTC	685 bp	
	R: TCGCTTATCAGGTGTGCTTG		
aphA1	F: TTATGCCTCTTCCGACCATC	489 bp	
	R: GAGAAAACTCACCGAGGCAG		
sul1	F: CGGCGTGGGCTACCTGAACG	433 bp	**Ibekwe *et al*., 2011**
	R: GCCGATCGCGTGAAGTTCCG		
***dfr*A**	F:TGGTAGCTATATCGAAGAATGGAGT	425 bp	**Grape *et al*. 2007**
	R:TATGTTAGAGGCGAAGTCTTGGGTA		

Molecular identification of antimicrobial resistance genes

Screening of the recovered isolates to the presence of AMR genes associated to tetracycline (*tetH*), ampicillin and penicillin (*bla*$_{ROB-1}$), aminoglycoside (*aphA1*), sulfa (sul1) and trimethoprim (*dfrA*) was performed by using PCR. The five pairs of primers were supplied from Metabion (Germany) or Biobasic (Canada) (table 2). The protocol included an initial denaturation step for 5 min at 94°C, followed by 35 cycles of three steps: second denaturation at 94°C for 30 sec, annealing of the primers at 60°C for 40 sec except *aph1* at 54°C for 40 sec and extension at 72°C for 45 sec, except *tetH* gene at 72 for 1 min. The final elongation takes place at 72°C for 10 min. After PCR, the reaction mixture was applied directly to a gel for analysis.

RESULTS

Isolation and identification of *P. multocida* from diseased chickens

Based on the phenotypic characterization nine *P. multocida* isolates (3.6%) have been recognized from the examined layer chickens. Four from lungs, two from liver and three from spleen, while no isolates were detected in broiler chickens.

Detection of capsular type A

Six isolates of *P. multocida* that were examined for detection *hya*D-*hya*C gene related with the capsular biosynthesis of serotype A were positive (Fig.1).

In vitro antimicrobial sensitivity test

P. multocida isolates showed 100% resistance to sulfamethoxazole and trimethoprim, nitrofurantion

Table 3. Antimicrobial Resistance Pattern of *P. Multocida* Isolated from Chicken lungs, spleen and Liver. (n = 9)

Number of isolates	Resistance phenotypic	Resistance to antimicrobial classes
1	RD, AMP, E, SXT,P, OX, SAM, OT, F, C	Eight classes
1	RD, K, SXT, P, OX, SAM, OT, F, CFP, C	Eight classes
1	RD, S, STX, OX, F, C, AK	Six classes
1	RD, E, STX, P, OX, SAM, F, C	Six classes
1	RD, SXT, P, OX, F, C	Five classes
1	RD, SXT, P, OX, SAM, F, C	Five classes
1	AMP, SXT, P, FD, OX, SAM, OT, F, TOB	Five classes
1	SXT, P, OX, SAM, F, C	Four classes
1	S, K, SXT, OX, AK, F, CFP, TOB, SAM	Four classes

*n = number of examined sample.
*AMR: antimicrobial resistance genes. Beta-lactams: P, penicillin10 IU; AMP, ampicillin 10 µg/mL; CTX cefotaxime 30 µg/mL, OX, Oxacillin 1 µg/mL, SAM, Ampicillin/sulbactam 20 µg/mL; tetracyclines: OT, oxy tetracycline 30 µg /mL; macrolides: E, erythromycin 15 µg/mL; folate pathway inhibitors: SXT, trimethoprim/sulfamethoxazole 25 µg /mL;aminoglycosides: TOB, tobramycin10 µg/mL, S, streptomycin 10 µg/mL; K, Kanamycin30 µg/mL; AK, Amikacin 30 µg/mL; phenicols: C, chloramphenicol 30 µg / mL; Nitrofurans: N, nitrofurantoin 300 µg/mL; Rifamycins, RD, rifampicin 30 µg/mL; third generation of cephalosprins: CFP, cefoperazone 75 µg/mL.

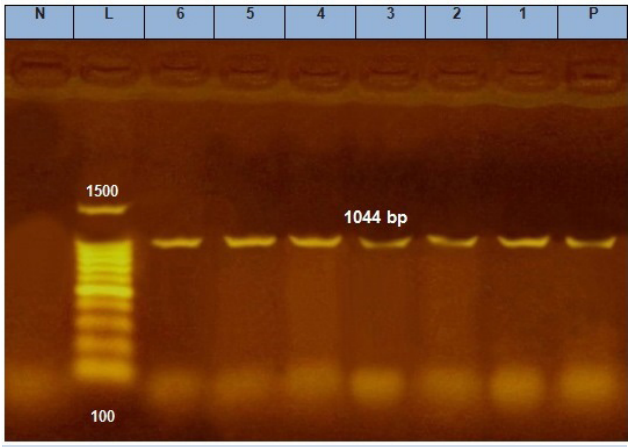

Figure 1. Amplified products of *P. multocida* by PCR assay .Lane L, 100 bp DNA ladder; lane P, positive control; lane N, negative control; lanes 1-6 *P. multocida* isolates positive for the capsular antigen type A at 1044 bp

and oxacillin, while the isolates were resistant to chloramphenicol, rifampicin, ampicillin/ sulbactam and penicillin in the percentage of 77.78 for each one and 33.3% for oxytertacycilline. On the other hand all isolates showed 100% sensitivity to cephalexin, clindamycin, norfluxacin, cefotaxime and polymyxinB, while 77.7% of the isolates were sensitive to streptomycin, ampicillin, kanamycin, erythromycin, cefoperazone, amikacin and tobramycin (table 3).

Molecular identification of AMR genes

Gene's *tetH*, $bla_{ROB-1,}$ *aphA1*, Sull and *dfrA* were analyzed by PCR. The predominant gene detected in all isolates (100%) was bla_{ROB-1} gene followed by sul1gene (50%), while *tet*H gene was detected in two isolates; moreover *dfrA* gene was detected in one isolate. (fig 2).

DISCUSSION

Fowl cholera is a highly contagious disease caused by *P. multocida* that affects a broadhost range of birds andcauseshigh mortality ratethat incurs significant economic losses in commercial and backyard poultry production (Christensenand Bisgaard, 2003). The incidence of fowl cholera along with other bacterial diseases is on the increase, despite vaccination and proper medication and can be attributed to various incriminating factors (Raji et al., 2010a).The clinical manifestations of avian pasteurellosis may cause high morbidity and mortality and occurs in several forms which can be pre acute, acute, chronic and localized disease (Glisson et al., 2008).

In this study incidence rate of *P.multocida* was 3.6%. This rate is higher those obtained by (Raji et al., 2010b) who reported that the prevalence rate of avian pasteurellosis in Zaria was 2.3 % and higher than reported by (Muhairwa et al., 2001; Raji*et al*., 2010a;Kwaga et al., 2013) who recorded that the incidence rate was (0.7, 1.5 and 1.2) respectively. On the other hand the highest incidence of *P. multocida* was reported by (Thulasi et al., 2013); (Panna et al., 2015); (Victor et al., 2016) and (Elalamy et al., 2020) in the percentage of (6.6, 11.4, 12.4 and 8), respectively.

The variation in the incidence among poultry in different governorates of the same country and in different countries depends on the differences in age and breeds of the chickens and also for the resistance power of the commercial chicken due to improved

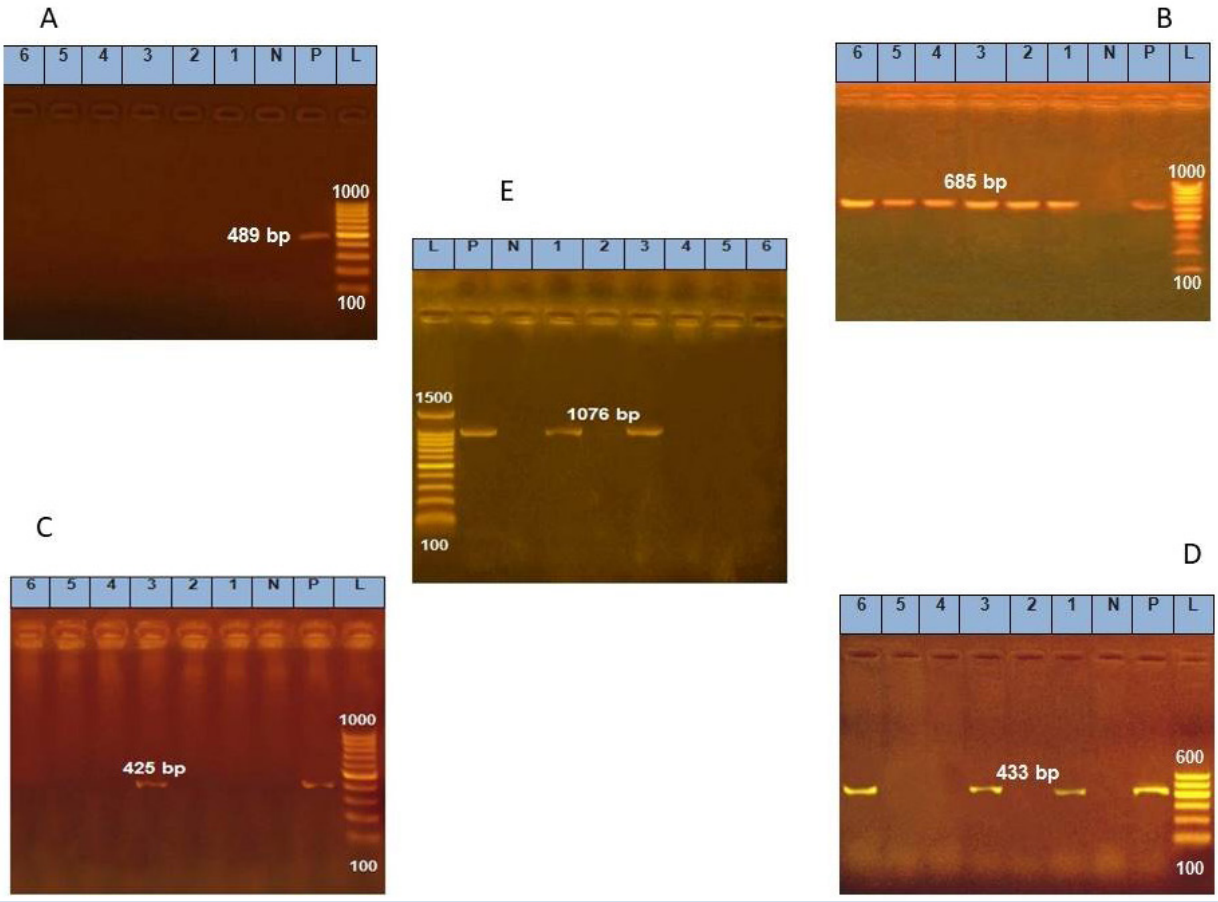

Figure 2. Show detection of bla_{ROB-1}, sul1,tetH, dfrA and aphA1in six strain of P.multocida.
A:Amplicons of aphA1 gene in six isolates of P.multocida. Lane L,100 bp DNA ladder; lane P, positive control; lane N, negative control; lane 1;6 P. multocida negative isolates for aphA gene at 489bp.
B: Amplicons of bla_{ROB-1} gene. Lane L,100 bp DNA ladder; lane P, positive control; lane N, negative control; lane 1:6, P. multocida positive isolates for bla_{ROB-1} gene at 685bp.
C:Amlicons of dfrA gene. Lane L,100 bp DNA ladder; lane P, positive control; lane N, negative control; lane 3 P. multocida positive isolates for dfrAgene at 425bp ;lane 1,2,4,5.6 negative isolates.
D: Detection of sul1gene. Lane L,100 bp DNA ladder; lane P, positive control; lane N, negative control; lane 1, 3, 6 P. multocida positive isolates for sul1 gene at 433bp ; lane 2,4,5 negative isolates.
E:Ampliconsof tetH gene. Lane L,100 bp DNA ladder; lane P, positive control; lane N, negative control; lane 1,3 P. multocida positive isolates for tetHgene at 1076bp ; lane 2,4,5.6 negative isolates

management number of samples, method of isolation, vaccine, nutrition and use of antimicrobials as prophylactic antimicrobial drugs.

Fowl cholera, which is generally caused by serotypes A is a severe systemic disease which occurs in domestic poultry and wild birds and results in significant economic losses to poultry industries worldwide. In this study the six isolated strains were examined and confined to be Type A by PCR.The similar results were reported by (Davies et al., 2003; Kwaga et al., 2013; Akhtar, 2013; Panna et al., 2015).

Antimicrobial treatment is still commonly utilized to control fowl cholera, but has been accompanied by the emergence of resistant strains. The resistant strains are a result of the widespread use of antimicrobials in feed for both prophylaxis and growth promotion. Antimicrobial resistance can develop in the strains by the molecular transmission of resistance mechanisms from other bacteria carried by mobile genetic elements (Tang et al., 2009).

Antimicrobial sensitivity test of all isolated *P. multocida* revealed that the organisms were 100% resistance to sulfamethoxazole and trimethoprim, nitrofurantion and oxacillin and this result is similar to that recorded by Shivachandra et al. (2006), Kwaga et al. (2013), Elalamy et al., (2020) and nearly similar to results which recorded by Zahoor and Siddique, (2006), Sarangi, and Panda (2011), Balakrishnan and Roy (2012) and Dashe et al. (2013), while the isolates

were resistant to ampicillin sulbactam, chloramphenicol, rifampicin and penicillin in a percentage of 77.78 for each one, the nearly similar to the observation also has been reported (Angrick et al., 2001; Rahman et al., 2004; Balakrishnan and Roy, 2012; Dashe et al., 2013), while (Elalamy et al., 2020; Kamruzzaman et al., 2016; Victor et al., 2016) had been recorded 100% resistance**.** 33.3% of the isolates demonstrated resistance to oxytertracycline and this result is accordance with that reported by Kamruzzaman et al., (2016), but Balakrishnan and Roy, (2012) recorded that 50% of isolates were sensitive. On the other hand all isolates showed 100% sensitivity to cephalexin, clindamycin, norfluxacin, cefotaxime and polymyxin B, while they were shown 77.78% sensitivity for streptomycin, ampicillin, kanamycin, erythromycin, cefoperazone, amikacin and tobramycin and this result is approximately similar to previous reported ones (Angrick et al., 2001; Shivachandra et al., 2006; Zahoor and Siddique, 2006; Sarangi and Panda, 2011; Balakrishnan and Roy, 2012;Furianet al.,2016; Kamruzzaman et al., 2016). These findings indicate that performing antibiotic sensitivity test is essential to control fowl cholera due to the emerging drug resistance in *P. multocida* (Panna et al., 2015).

The multidrug resistance occurs when single bacteriumis resistant to at least one antimicrobial drug in three or more antimicrobial classes of antibiotic. The MDR transfer and spread among bacteria through plasmid which carry many resistant gene, so the MDR bacteria has public health threat and difficult for treat (Magiorakos et al., 2012). In our study, (2, 1, 2, 2, 2) were resist to (10, 9, 6, 8, 7) different antimicrobial drugs in different classes. This result imitates those recorded by Elalamy et al. (2020) and Furian et al. (2016).

In parallel with the phenotypic antibiotic resistance testing, we also investigated the prevalence ofspecial resistance genes for Beta-lactams, aminoglycoside, sulfa, trimethoprim and tetracycline which are considered critically important antimicrobials to veterinary medicine as categorized by the World Organization for Animal Health (OIE, 2014). The results showed that six examined strains carried at least one resistance gene tested indicating that these genes have a major role in conferring resistance among the strains investigated.

The bla_{ROB-1} gene, which widespread in *Pasteurellaceae* family was found in all examined strains. This result is similar to that recorded by San Millan et al., (2009) and Dayao et al., (2016) but differ than others (Elalamy et al., 2020), who found bla_{ROB-1} in the percentage of 8.3. Finding of bla_{ROB-1}genein the tested isolates suggests that ampicillin and penicillin resistance is most likely caused by the β-lactamase enzyme.

The gene *tetH* that confer tetracycline resistance, was originally detected in an avian *P. multocida* isolate from the USA (Kehrenberg et al., 2001), but later were found that tetracycline genes are often associated with conjugative and mobile genetic elements (plasmids or transposons), which permit the horizontal transfer of the tetracycline resistance genes between receptive strains (Dayao et al., 2016). Kehrenberg and Schwarz, (2000) found that the *tetH*gene most frequently seen in *Pasteurella* isolates. In this study *tetH* gene was detected in the percentage of 33.3 a higher percentage than those of Klima et al., (2014) and Oh et al., (2019) who detected *tetH* gene in the percentage of 18 and16.2 respectively. However, Babetsa et al., (2012) and Elalamy et al., (2020) detected *tetH* gene in the percentage of 100 and 72.2 respectively. The presence of *tetH* suggested that the underlying mechanism of decreased susceptibility and resistance to tetracycline is related to the action of efflux pump proteins that expel the drugs out of the cell leading to inactivity of tetracycline against the bacterial pathogen (Dayao et al., 2016).

Sulfonamide resistance is one of the most often detected resistance properties among Pasteurella isolates (Kehrenberg et al., 2001). In our study, sul1 gene detected in the percentage of 50, while Olonitola et al. (2015)detected sul1 gene in all isolates which showed resistant to sulfonamide,Moreover they also detected it from some isolates showed sensitive to sulfonamide. This indicates that antimicrobial genes may be present, but inactive.

The *dfrA* gene that confers to trimethoprim resistance was detected in this study in the percentage of 16.6. Escande et al. (1991) reviewed that previous attempts to identify resistance trimethoprim gene in bacteria of the genus Pasteurella have failed so Kehrenberg et al. (2001)assumed that bacteria of the genus *Pasteurella* may carry *dfrA* gene different from this previously identified in other gram-negative bacteria. The finding of *dfrA* in isolates means that trimethoprim resistance happened due to modification in the gene coding dihydrofolate reductase (Al-Assil and Hamzeh, 2013).

CONFLICT OF INTEREST

None declared.

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