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Original Research

RT-PCR detection of exotoxin genes expression in multidrug resistant *Pseudomonas aeruginosa*

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Abstract: *Pseudomonas aeruginosa* (PA) is an opportunistic pathogen responsible for causing a wide variety of acute and chronic infections with significant levels of morbidity and mortality. These infections are very hard to eradicate because of the expression of numerous virulence factors and the intrinsic resistance against antibiotics. Herein, this study analyzed antimicrobial susceptibility of PA isolated from broiler chickens and cattle as well as expression of five significant exotoxin genes (*exo*U, *exo*S, *tox*A, *las*B, and *phz*M) and *ecf*X as internal control. Genomic DNA was amplified employing *opr*L gene for species specific detection of PA. The highest resistance was found to ampicillin, erythromycin, followed by, chloramphenicol, trimethoprim/ sulfamethoxazole and tetracycline, intermediately sensitive to ceftazidime, cefoperazone, and highly sensitive to gentamicin, levofloxacin, imipenem, ciprofloxacin and colistin. It appears that *exo*U$^+$ and increased resistance to SXT may be co-selected traits. Vast majority of PA isolates expressed *exo*S (78.6%), *exo*U (71.4%) and both in more virulent strains. The ubiquity of *tox*A, *las*B, *exo*U and *exo*S among PA clinical isolates is consistent with an important role for these virulence factors in chicken respiratory diseases and cattle mastitis that can be highlighted as potential therapeutic targets for treatment of infections caused by heterogeneous and resistant PA strains.

Key words: *P. aeruginosa*, Antimicrobial susceptibility, Gene expression, Exotoxin genes, *opr*L gene.

Introduction

Pseudomonas aeruginosa is an opportunistic, Gram-negative bacillus that causes a variety of clinically important infections in compromised hosts and in the critically ill, which are most commonly involved in patients with cystic fibrosis, severe burns, neutropenia, and the mechanically ventilated (1). *P. aeruginosa* mastitis in dairy cattle is frequently owing to the use of infected rinsing water (2). It is not surprising that these illnesses are associated with significant morbidity and mortality due to the organism's capacity to adapt easily to changes in the environment, to rapidly develop resistance to antibiotics, and to produce an arsenal of virulence factors (3). Exotoxin A (ETA), pyocyanin, elastase, and type III secretion system are the most significant virulence determinants of *P. aeruginosa* and cause great concern (4). ETA, an important ADP- ribosyltransferase toxin that encoded by *tox*A gene (5); pyocyanin, the most studied phenazine with redox capabilities leading to host cell damage (6,7), conversion of phenazine-1-carboxylic acid to pyocyanin is mediated by *phz*M gene (8); elastase that encoded by *las*B gene, is a protease that can degrade host defense, immunoregulatory proteins and damage epithelia (9,10) and type III secretion system, a system that can inject toxic effector proteins into the cytoplasm of eukaryotic cells (11). To date, four effector proteins have been described in *P. aeruginosa*: *exo*U, *exo*S, *exo*T, and *exo*Y (12,13). *Exo*T and *exo*Y have minor effect on virulence, while *exo*U and *exo*S have great contribution to pathogenesis and cause great concern (14). In addition, *ecf*X gene encodes an ECF (*e*xtra-*c*ytoplasmic *f*unction) sigma factor which is restricted to *P. aeruginosa*, might play a role in haem-uptake and virulence and has been described as an internal amplification control gene (4,15,16). *P. aeruginosa* is noted for its metabolic versatility and its exceptional ability to colonize a wide variety of environments and also for its intrinsic resistance to a wide variety of antimicrobial agents. Because of its virulence and the limited choices of effective antimicrobial agents, treatments of infections by *P. aeruginosa* are often difficult(17). The emergence of multidrug-resistant (MDR) *P. aeruginosa* strains is a serious problem (18). The knowledge of the frequency and susceptibility pattern of the MDR *P. aeruginosa* is very important for our clinicians in order to better treat their patients (19).

Interestingly, *P. aeruginosa* in various environments has high genotypic diversity, leading to deficiency of exotoxin genes for some strains(15,20,21). The distribution of these genes amongst clinical isolates of *P. aeruginosa* remains to be elucidated. Furthermore, the frequency of effector genes in populations of isolates from different disease sites has not been thoroughly examined (20). For that reasons, the present study has been carried out to study the expression of five virulence genes (*tox*A, *phz*M, *las*B, *exo*U and *exo*S) of *P. aeruginosa* isolates recovered from broiler chicken respiratory diseases and cattle mastitis as well as the antimicrobial susceptibility to commonly used antimicrobial agents. To the best of our knowledge, this work have rarely been documented in Egypt.

Materials and Methods

Clinical isolates

This study was performed on *P. aeruginosa* clinical isolates (n=14) that were obtained from broiler chic-

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kens and dairy cattle. Bacteria were isolated from lung, trachea of chickens suffered from respiratory diseases and cattle mastitic milk. All isolates were identified by conventional tests(22). Identification analysis like Gram staining, motility, catalase, oxidase, pigment production, growth on cetrimide agar, haemolysis on blood agar, ability to grow at 42◦C, gelatin hydrolysis and nitrate reduction tests were carried out. The 20% glycerol stocks of all isolates were stored at –70 °C until studied.

Antimicrobial susceptibility test

Antibiogram of *P. aeruginosa* isolates for 12 commercially available antibiotic discs (Hi-Media, Mumbai) was determined by Kirby Bauer disc diffusion method. Suspensions of the isolates of 0.5 McFarland turbidity standard were made and Mueller Hinton Agar (MHA) plates were inoculated. Antibiotic discs of ampicillin (10 µg), erythromycin (10 µg) tetracycline (30 µg) imipenem (10 µg), gentamicin (10 µg), trimethoprim / sulfamethoxazole (25 µg), colistin (10 µg), cefoperazone (75 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg) and chloramphenicol (10 µg) were applied on the plates. The plates were then incubated at 37°C for 24 hours and the results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (23).

Genotypic characterization of the isolates

PCR for oprL gene

PCR for *opr*L gene was performed following extraction of genomic DNA using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Strains were cultured in brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) and incubated under aerobic conditions at 37 °C for 24 h. Aliquots (200 µl) of bacterial suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. To measure the quantity and purity of genomic DNA, NanoDrop ND- 1000 spectrophotometer (NanoDrop, USA) was used.

RT-PCR for virulence gene expression

Isolates were confirmed as *P. aeruginosa* by presence of *opr*L gene were used to screen for the expression of five significant enterotoxin genes (*tox*A, *phz*M, *las*B, *Exo*U and *Exo*S) and one internal control gene *ecf*X. The oligonucleotide primers and cycling conditions used in this study are listed in Table 1. For RNA extraction a double volume (1 ml) of the RNA protect bacteria reagent (Qiagen, Germany, GmbH), was added to one volume (0.5 ml) of the broth of the harvested culture to protect RNA from degradation, the mix was then vortexed and incubated for 5 min. at room temprature, centrifugated for 10 min. at 8000 rpm. The supernatant was decanted. Then 200 µl of TE buffer containing 1 mg/ml Lysozyme (Biochemica, Applichem) and 700 µl RLT buffer containing 10 µl β-mercaptoethanol per 1 ml were added to the pellet. Then 500 µl of 100% ethanol was added, and the steps was completed according to the enzymatic lysis of bacteria protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). To remove residual DNA DNase digestion was done on column of the kit.

All PCR reactions were done on a total volume of 25 µl reaction containing 5 µl of DNA template, 12.5 µl of EmeraldAmp® Max PCR Master Mix (Takara, Japan), 1 µl of each primer (20 pmol concentration) and 5.5 µl of nuclease free water. PCR amplification was performed in a thermal cycler (Biometra T3 Thermocycler, Göttingen, Germany).The amplified products were electrophoresed on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature, stained with 0.5µg/mL ethidium bromide. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes (Table 1).The gel was photographed by a GelDoc UV gel documentation system

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions for PCR.

Target gene	Primers sequences (5′–3′)	Amplicon size (bp)	Reverse transcription	Initial denaturation	Amplification (35 cycles)			Final extension	Reference
					Denaturation	Annealing	Extension		
*tox*A	F GACAACGCCCTCAGCATCACCAGC R CGCTGGCCCATTCGCTCCAGCGCT	396		94°C 10 min.	94°C 45 sec.	55°C 45 sec.	72°C 45 sec.	72°C 10 min.	(24)
*exo*S	F GCGAGGTCAGCAGAGTATCG R TTCGGCGTCACTGTGGATGC	118		94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	72°C 7 min.	(25)
*exo*U	F CCGTTGTGGTGCCGTTGAAG R CCAGATGTTCACCGACTCGC	134		94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	72°C 7 min.	
*ecf*X	F ATGGATGAGCGCTTCCGTG R TCATCCTTCGCCTCCCTG	528	50°C 30 min.	94°C 10 min.	94°C 45 sec.	50°C 45 sec.	72°C 45 sec.	72°C 10 min.	(26)
*Las*B	F ACAGGTAGAACGCACGGTTG R GATCGACGTGTCCAAACTCC	1220		94°C 15 min.	94°C 1 min.	54°C 1 min.	72°C 1.5 min.	72°C 12 min.	(27)
*phz*M	F ATGGAGAGCGGGATCGACAG R ATGCGGGTTTCCATCGGCAG	875		94°C 10 min.	94°C 1 min.	54°C 1 min.	72°C 1 min.	72°C 10 min.	
*opr*L	F ATG GAA ATG CTG AAA TTC GGC R CTT CTT CAG CTC GAC GCG ACG	504	-	94°C 10 min.	94°C 45 sec.	55°C 45 sec.	72°C 45 sec.	72°C 10 min.	(28)

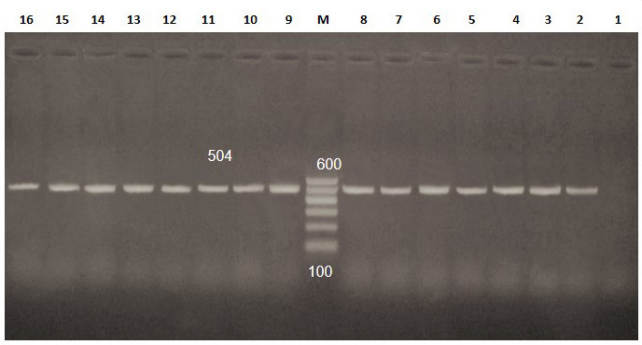

Figure 1. PCR amplicons of *P. aeruginosa opr*L gene. Lane M, 100-bp ladder; lane 1, negative control; lane 9, positive control; lanes 2–16, positive isolates at 504bp amplicons.

(Alpha Innotech, Biometra).

Statistical analysis

STATA/MP, version 13.1 (StataCorp LP, College Station, TX) and with GraphPad Prism (La Jolla, CA) SPSS, version 21 for Windows; SPSS, Chicago, IL software was used for data management and statistical analysis. Binary logistic regression was used to study the association between the expression of certain virulence genes and resistance to antibiotics with an odds ratios (OR) indicating the strength of the associations. Associations of interest with a zero in the contingency table were noted and evaluated for significance with a two-sided Fisher exact test (significant at a *P* value of < 0.05).

Results

Regarding the susceptibility profile of *P. aeruginosa* clinical isolates to different antibiotics, the highest resistance was found with ampicillin, erythromycin (100% for each), followed by, chloramphenicol (78.6%), trimethoprim/ sulfamethoxazole (71.4%) and tetracycline (64.3%), intermediately sensitive to ceftazidime (57.1%), cefoperazone (5o%), and highly sensitive to gentamicin, levofloxacin, imipenem, ciprofloxacin (100% for each), and colistin (92.9%). PCR amplification of *opr*L gene of all *P. aeruginosa* isolates showed amplified product at 504bp. (Fig.1). Transcription of *tox*A occurred in all 14 examined MDR *P. aeruginosa* isolates and elastase gene (*las*B) in 12 isolates that demonstrated by the production of a 396 and 1220 bp amplicons respectively (Fig. 2A, 2B). RT-PCR for *Phz*M gene demonstrated by the production of 875 bp amplicons that were not detected in all clinical isolates. Seven out of 14 examined *P. aeruginosa* isolates from chicken and cattle were positive for the expression of both genes *exo*U and *exo*S. Clinical isolates encoding only the *exo*S cytotoxin gene (n=4) were more than those encoding only *exo*U (n=3) as revealed in table (2) and Fig.2C, 2D. *Ecf*X gene was used as internal control that yield amplicons at 528 bp. The OR could not be estimated for six antibiotics which have 100% of sensitivity and resistance for *P. aeruginosa* isolates. An association was found between the expression of *exo*U gene and SXT resistance (p-value = 0.041). Expression of other virulence genes was not associated with antibiotic resistance (p-value > 0.05) as in table (3). The odds of identifying *exo*U and SXT resistant isolates were 27 times higher in resistance than isolates that not expressed gene. Although, *exo*U gene expression and TE resistance association was non-significant on nonparametric analysis (P = 0.095), the odds of *exo*U and TE resistance

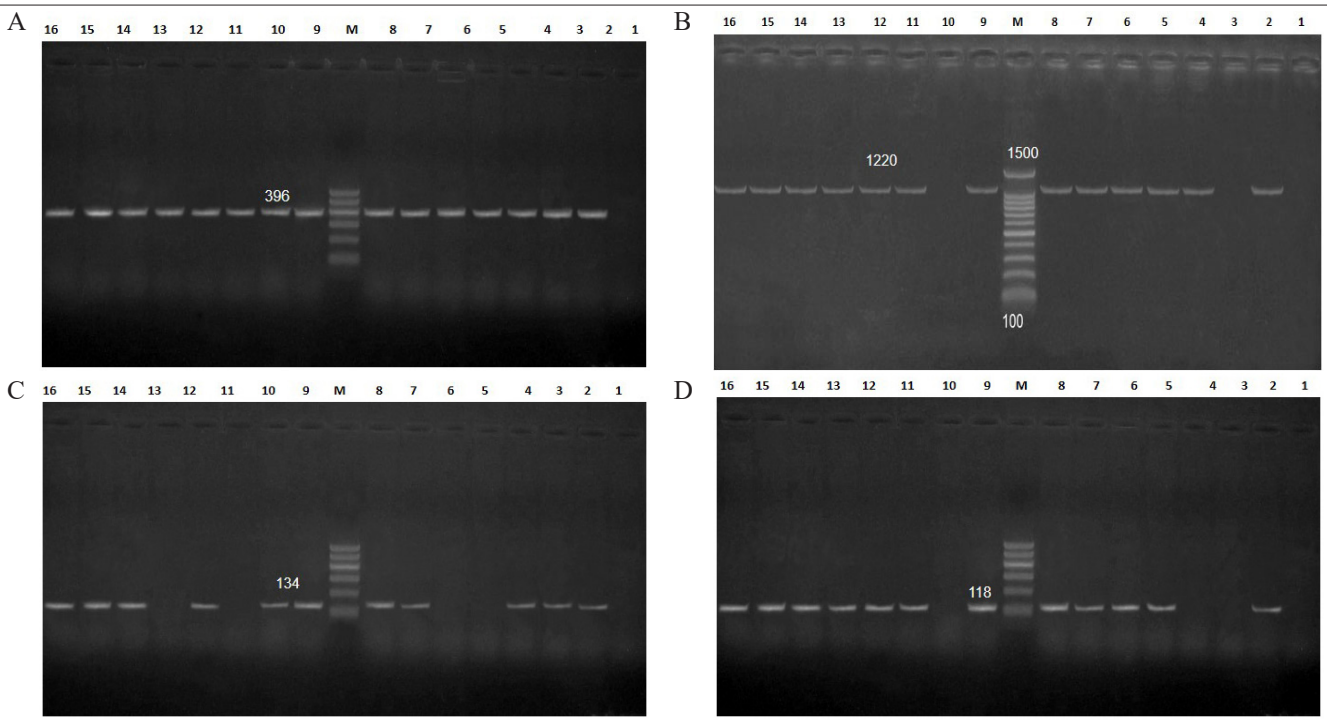

Figure 2. RT-PCR amplicons of *P. aeruginosa* exotoxin genes. **A:** Expression of *tox*A gene. Lane M, 100-bp ladder; lane 1, negative control; lane 9, positive control; lanes 2–16, positive isolates at 396bp amplicons. **B:** Expression of *las*B gene. Lane M, 100-bp ladder; lane 1, negative control; lane 9, positive control; lanes 2, 4–8, 11-16, *P. aeruginosa* isolates positive for gene expression at 1220bp; lanes 3, 10, negative isolates. **C:** Expression of *exo*U gene. Lane M, 100-bp ladder; lane 1, negative control; lane 9, positive control; lanes 2-4, 7,8,10, 12, 14-16, *P. aeruginosa* isolates positive for gene expression at 134bp; lanes 5,6,11, 13, negative isolates. **D:** Expression of *exo*S gene. Lane M, 100-bp ladder; lane 1, negative control; lane 9, positive control; lanes 2, 5–8, 11-16, *P. aeruginosa* isolates positive for gene expression at 118 bp; lanes 3,4,10, negative isolates.

Table 2. Resistance pattern and virulence genes expression of MDR *P. aeuriginosa* strains.

Source /Strain No.	Resistance pattern*	Virulence genes				
		***exo*U**	***exo*S**	***las*B**	***tox*A**	***phz*M**
Chicken 1	TE, AMP, E, C, SXT CAZ	+	+	+	+	-
2	TE, AMP, E, C, SXT	+	-	-	+	-
3	TE, AMP, E, C, SXT	+	-	+	+	-
4	AMP, E, C, CAZ	-	+	+	+	+
5	TE, AMP, E, C, SXT	-	+	+	+	+
6	TE, AMP, E, C, SXT	+	+	+	+	-
7	AMP, E, C, SXT	+	+	+	+	-
8	TE, AMP, E, C, SXT	+	-	-	+	-
9	AMP, E, C, CAZ,CEP	-	+	+	+	-
10	AMP, E, CAZ,CEP	+	+	+	+	-
Cattle 11	AMP, E, C	-	+	+	+	-
12	TE, AMP, E, SXT	+	+	+	+	-
13	TE, AMP, E, SXT	+	+	+	+	-
14	TE, AMP, E, C, SXT, CEP	+	+	+	+	-

TE, tetracycline; AMP, ampicillin; E, erythromycin; C, chloramphenicol; SXT, trimethoprim - sulfamethoxazole; CEP, cefoperazone; CAZ, ceftazidime; CIP, ciprofloxacin.

Table 3. Significant P-value of Fisher's exact test for the association of virulence gene and antibiotic resistance.

Virulence gene	Antibiotic resistant and virulence gene positive isolates	OR	P-value*
***exo*U**	TE 8 (80.0%)	12	0.095
	C 7 (70.0%)	NaN*	0.506
	SXT 9 (90.0%)	27.00	0.041
	CEP 2 (20.0%)	0.75	0.999
	CAZ 2 (16.7%)	0.4	0.5686
	CT 1 (9.1%)	1.62E+08	0.999
***exo*S**	TE 6 (54.5%)	NaN	0.258
	C 8 (72.7%)	NaN	0.547
	SXT 7 (63.6%)	NaN	0.506
	CEP 3 (27.3%)	6.1E+08	0.547
	CAZ 4 (26.7%)	5.9E+08	0.999
	CT 1 (8.3%)	1.47E+08	0.999
***las*B**	TE 7 (58.3%)	NaN	0.505
	C 9 (75.0%)	NaN	0.999
	SXT 8 (66.7%)	NaN	0.560
	CEP 3 (25.0%)	5.4E+08	0.999
	CAZ 4 (25.0%)	5.4E+08	0.999
	CT 1 (7.7%)	1.35E+08	0.999
***tox*A**	TE 9 (64.3%)	NaN	-
	C 11 (78.6%)	NaN	-
	SXT 10 (71.4%)	NaN	-
	CEP 3 (21.4%)	NaN	-
	CAZ 4 (22.2%)	NaN	-
	CT 1 (6.7%)	NaN	-
***phz*M**	TE 1 (50.0%)	0.5	0.999
	C 2 (100.0%)	4.4E+08	0.999
	SXT 1 (50.0%)	0.33	0.999
	CEP 0 (0.0%)	NaN	0.999
	CAZ 1 (33.3%)	2	0.999
	CT 0 (0.0%)	NaN	0.999

Fisher Exact Probability Test (Two-tailed); OR: odds ratio; NaN: the OR could not be estimated.

combination was 12 times higher than isolates that not expressed gene. Similarly, the odds of CAZ resistance in isolates that expressed *phz*M was increased one times than in *phz*M negative isolates. While, the odds of association *phz*M and TE resistance was decreased 0.5 times than negative isolates.

Discussion

P. aeruginosa success as a dangerous and dreaded pathogen comes from its genetic/metabolic plasticity, intrinsic/acquired antimicrobial resistance, capacity to form biofilm and expression of numerous virulence factors(29). This study investigates the expression of five significant virulence genes among MDR *P. aeruginosa* clinical isolates. All those isolates of *P. aeruginosa*, which are resistant to at least one anti-microbial agent in three or more anti-pseudomonal anti-microbial categories are termed as multi-drug resistant (MDR). While, those isolates which are resistant to at least one antimicrobial agent in six or more anti-pseudomonal antimicrobial categories are defined as extremely drug resistant (XDR) (30) In the present study, twelve antimicroloial agents were tested against *P. aeruginosa* clinical isolate. The reason choosing this antimicrobials was their wide use as anti-psedudomonal agents. Therefore, this study could provide appropriate guidelines to cattle and chicken farms regarding the prescription of these antimicrobials according to their sensitivity to *P. aeruginosa***.** Our findings document five antimicrobial agents, gentamicin, ciprofloxacin, levofloxacin, imipenem and colistin, show potent antibacterial activity against *P. aeruginosa* strains. While, out of 14 *P. aeruginosa* isolates, 12 were MDR (resistant to 3 and more antimicrobial agents in anti-pseudomonal antimicrobial catagories) and two were considered as XDR (resistant to six antimicrobials).Similarly, Aloush et al. (31) have reported an incidence of 14 MDR strains of *P. aeruginosa* per 10,000 hospital admissions. In another study conducted in Japan, Kirikae et al. (32) revealed an incidence of 1.1% of MDR *P. aeruginosa* while, defined strains resistant to carbapenems, fluoroquinolones and amikacin as MDR. However, Vosahlikova et al.(21) found that all *P. aeruginosa* isolates recovered from cystic fibrosis (CF) patients were susceptible to colistin; 94–96% were susceptible to piperacillin, ceftazidime, cefepime, meropenem, amikacin or tobramycin; and 84–87% were susceptible to ciprofloxacin and gentamicin. In another study conducted in India, ciprofloxacin and cefotaxime were found to be better choices for treating *P. aeruginosa* infections (33). In accordance with earlier observations (34,35) colistin is a reliable solution in cases of infections with MDR, XDR *P. aeruginosa***.** In addition, the current study has demonstrated that *P. aeruginosa* strains were sensitive to imipenem and this is in agreement with Livermore (36) that a carbapenems have a broad antibacterial spectrum and play a fundamental role in the treatment of infections caused by MDR *P. aeruginosa* isolates. Nevertheless, it was reported that resistance to imipenem was 14% in Spain, 19.3% in Italy and 68% in Saudi Arabia (17,37,38).

The present study has shown the highest resistance of *P. aeruginosa* isolates were with ampicillin, erythromycin, followed by, chloramphenicol, trimethoprim/sulfamethoxazole and tetracycline and intermediately sensitive to ceftazidime, cefoperazone. These findings are in agreement with previous reports, which had concluded that *P. aeruginosa* isolates were resistant to ampicillin, erythromycin and tetracycline(33,39). On the other hand, isolates recovered from cattle in Bangladesh were sensitive to chloramphenicol and intermediately sensitive to kanamycin, erythromycin, gentamicin and sulfamethoxazole(40). Whether MDR *P. aeruginosa* strains necessarily express a more virulent phenotype continues to remain a controversial issue (41). The present study revealed that *tox*A gene was found in each of 14 examined MDR *P. aeruginosa* isolates, suggesting that this gene is not a variable trait, and *las*B gene in 12 isolates. These results agree with previous observations in which each of 32 *P. aeruginosa* examined isolates carried and transcribed the potent virulence factor, exotoxin A gene by using PCR and RT-PCR(24). There is an inversely proportional correlation between the exoprotein levels including *las*B, *alg*D, and *tox*A and clinical improvement in chronically infected CF patients(42). However, it was demonstrated that *las*B is transcribed more frequently and at a higher level than *tox*A in CF patients with *P. aeruginosa* infections(9).

The role of *tox*A and *las*B gene produced by *P. aeruginosa* in cattle mastitis and the severity of symptoms have not been previously investigated. Interestingly, the presence of these two genes in all examined clinical isolates with the exception of two isolates were *Las*B negative suggests that *tox*A and *Las*B may play a role in the respiratory tract infection of chickens and cattle mastitis. Unlike *tox*A and *las*B, *phz*M gene were not found in all clinical isolates. Similarly, Shi et al. (4) not detected *phz*M in all tested strains. These observations concur with previous studies, in which *P. aeruginosa* clinical isolates show high genotypic diversity (21), and several studies have verified the deficiency of these exotoxin genes in some *P. aeruginosa* strains (15,20). Not only is *phz*M clearly involved in the biosynthesis of pyocyanin, but there is a complex biosynthetic pathway in *P. aeruginosa* that there are two core loci responsible for synthesis of phenazine-1-carboxylic acid (PCA) and three additional genes (*phz*M, *phz*S, and *phz*H) encoding unique enzymes involved in the conversion of PCA to redox-active phenazine compounds including, pyocyanin, 1-hydroxyphenazine, and phenazine-1-carboxamide. *P. aeruginosa* with insertionally inactivated *phz*M or *phz*S developed pyocyanin-deficient phenotypes(8). It was reported that the vast majority of the examined *P. aeruginosa* isolates contained both the *exo*U and *exo*S genes that involved in phagocytosis and lung injury(27). In agreement with this finding, our study showed that 7 out of 14 examined strains contained both genes, so those strains may be the most virulent strains. Meanwhile, others have found that the vast majority of *P. aeruginosa* isolates contained either *exo*S or *exo*U but not both (14,43). It is known that 90% of *exo*U-producing *P. aeruginosa* strains are associated with severe infections. Secretion of *exo*U gene is a marker for highly virulent *P. aeruginosa* isolates obtained from pneumonic patients that may require more aggressive therapy than those infected with strains that do not secrete this toxin. (14). The present study revealed that most *P. aeruginosa* respiratory isolates (7/10)

were *exo*U+. In contrast, it was reported that 25–30% of respiratory isolates carried *exo*U(43). These data suggest that *exo*U$^+$ and trimethoprim-sulfamethoxazole resistance may be co-selected traits that result in highly virulent and resistant strains. It is possible that more virulent *exo*U-containing isolates caused more disease symptoms, and were thus exposed to more SXT, thereby giving rise to the emergence of resistance. Strateva et al. ascertained that the isolates containing the *exo*U gene were significantly more resistant (P<0.05) to different classes of antimicrobials(20) In agreement with Shi et al (4)**,** our findings showed that more clinical isolates carried *exo*S than *exo*U. Concomitantly, it was suggested that the actual overall prevalence of the *exo*S gene in clinical isolates was approximately 70%(43).*Opr*L is an outer membrane lipoprotein which has been implicated in efflux transport systems, as well as affecting cell permeability. PCR targeting the *opr*L gene, is a useful technique in the detection of *P. aeruginosa* (29). *Opr*L psitive *P. aeruginosa* strains were further tested for the expression of five virulence genes and *ecf*X gene as internal amplification control that has been previously described in another studies (4,15,16).

The ubiquity of *tox*A, *las*B and type III effector proteins encoding genes (*exo*U and *exo*S) among MDR *P. aeruginosa* clinical isolates is consistent with an important role for these virulence attributes in chicken respiratory diseases and cattle mastitis. Gentamicin, levofloxacin, imipenem, ciprofloxacin and colistin are the best drug of choice for the treatment of *P. aeruginosa* infection in this small scale study.

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