Research Article

Pseudomonas Species Isolated from Camels: Phenotypic, Genotypic and Antimicrobial Profile

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Abstract | One of the important livestock economies are camels which adapt with adverse environmental conditions and provide milk, meat, wool, hides, and skin. Gram-negative *Pseudomonas aeruginosa* is harbor multidrug antimicrobial resistance of camel has serious consequences for human health, so, this study aimed to characterized of *P. aeruginosa* especially extended spectrum β-lactamases (ESBL) producing one; phenotypic and genotypic. The isolates of *P. aeruginosa* were confirmed biochemically by a Vitek 2 compact system (bioMe´rieux). Suspected *P. aeruginosa* colonies carry ESBL were 56.6% by the double disc synergy test (DDST). Detection of virulence genes using *pslA*, *toxA* and *exoU* genes revealed that 29.4%, 23.5% and 17.6% respectively. Molecular detection of ESBLs encoding genes in *P. aeruginosa* recorded *that bla*TEM genes *bla*SHV *and bla*CTXM genes were detected in percentages of 64.7%, 47.0 % and 29.4%, respectively. Finally, ESBL *P. aeruginosa* showing multidrug antimicrobial resistance that detected by *mexR* gene.

Keywords | Camels, *P. aeruginosa*, ESBL, Antibiotic resistance, Virulence genes

Received | August 19, 2021; **Accepted** | October 05, 2021; **Published** | January 04, 2022
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Citation | Abdelrahman AM, Mohamed SR, Soliman SM, Marouf S (2022). Pseudomonas species isolated from camels: Phenotypic, genotypic and antimicrobial profile. Adv. Anim. Vet. Sci. 10(2): 219-225.
DOI | http://dx.doi.org/10.17582/journal.aavs/2022/10.2.219.225
ISSN (Online) | 2307-8316

INTRODUCTION

In Middle East region, camels are important in the livestock economy by naturally resistant to adverse environmental conditions and most of the diseases commonly affecting livestock (Ismail et al., 2014). camels are the main source of meat and milk in many regions of the world, mainly in Africa and Asia, playing a crucial role in their economy. Therefore, as they are important food sources in semi-arid and arid zones the picture of dromedaries transformed from "ship of the desert" to a "food security livestock" species and the camel industry is in transition from nomadism to intensive production. Although this trend recognizes the economic value of this livestock species as a food source, it could also make camels an increasingly important source for zoonotic disease transmission to humans, especially in resource poor communities with improper sanitation and medical access (FAO, 2019).

Pseudomonas aeruginosa causes different diseases in both livestock and companion animals as endometritis, otitis, hemorrhagic pneumonia, mastitis and urinary tract infections (Salomonsen et al., 2013). Bacteria adapt and acquire resistance from misuse and overuse of antibiotics in treatment of human illness, animal husbandry and antibiotics residues in agriculture leaves (CheeSanford et al., 2009). The increasing resistance of potentially pathogenic bacteria to multiple conventional antibiotics is an urgent problem in global public health (Strauß et al., 2015). The multiple-drug-resistant (MDR) *Pseudomonas* can be transmitted from different sources to humans and also to the environment through horizontal gene, the emergence and occurrence of MDR *P. aeruginosa* strains are growing in the world, leading to limited therapeutic

options (Breidenstein et al., 2011). Transmission of ESBL-producing gram-negative bacteria between food-producing animals and humans via direct contact or meat is supposed (Smet et al., 2010).

As few knowledges is available about *P. aeruginosa* in camel, this study aimed to investigate the ESBL producing *P. aeruginosa* from apparent healthy and diseased camels especially as this microorganism has the ability of producing multidrug resistant enzymes that could be easily disseminated in the community between livestock.

MATERIALS AND METHODS

Samples

Two hundred and fifty nasal swabs collected from apparent healthy (150) and diseased camels (100) at different Cairo and Giza farms and abattoirs, then sent to the laboratory on the ice box for bacterial examination.

Cultivation and isolation of *P. aeruginosa*

Following culture of samples on cetrimide agar, the plates were incubated aerobically at 37°C for 24 hours. The Suspected colonies were picked up for morphological and biochemical identification (Quinn et al., 2004) as traditional method of identification and reinvestigated biochemically by Vitek2 compact system according to the manufacture structure (Biome'rieux, 2006; Sahar et al., 2014).

Differentiation between ESBL and non ESBL by double disk synergy test method (DDST)

P. aeruginosa isolates were phenotypically identified as ESBL by double disk synergy test as described by (Jarlier et al., 1988) method. A Mueller–Hinton agar was inoculated with standardized inoculum (corresponding to 0.5 McFarland tube) using a sterile cotton swab, then an amoxicillin clavulanic acid (AMC 30 µg) disk placed in the center of the plate 15 mm away from ceftriaxone (CRO 30 µg), ceftazidime (CAZ 30 µg), cefotaxime (CTX 30 µg) and aztreonam (ATM 30 µg). The plate was incubated at 37 °C overnight. So, enhancement of the zone of inhibition of any one of the four drug disks toward amoxicillin–clavulanic acid suggested identification of extended-spectrum beta-lactamases (ESBL). *P. aeruginosa* ATCC 27853 was used as a control strain for a positive ESBL.

Antimicrobial resistance test

Antimicrobial resistance test was conducted on ESBL producing *P. aeruginosa* strains using the Kirby-Bauer disk diffusion method (Bauer et al., 1966) by using Mueller–Hinton agar plates the antimicrobial susceptibility are measured according to the standard procedures of Clinical and Laboratory Standards Institute guidelines CLSI (2020). the antimicrobial susceptibility of *P. aeruginosa* isolates was tested against different antimicrobial drugs of different classes: β-lactemas e.g Penicillin G (P 10µg), aztreonam (AT 30 µg), 3rd generation cephalosporin e.g cefotaxime (CTX30 µg), 4th generation cefepime (FEP 30 µg), Carbapenems e.g., imipenem (IPM 10 µg), meropenem (MEM 1 µg), Aminoglycosides e.g., gentamicin (GEN 10 µg), Quinolones e.g., ofloxacin (OFX 5 µg), Macrolides e.g., erythromycin (E15µg) and Sulfonamides e.g sulphamethoxazole/ trimethoprim (SXT 25 µg). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality controls.

Molecular identification of *P. aeruginosa*, virulence genes, ESBLs encoding genes and multidrug resistance gene by Polymerase Chain Reaction (PCR)

DNA from samples was extracted using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°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.

Primers used were supplied from Metabion (Germany) to detect *P. aeruginosa 16S rDNA,* ESBLs encoding genes (*bla*TEM, *bla*SHV and *bla*CTXM), virulence genes (*tox*A, *exo*U, and *psl*A) and multidrug resistance gene (*mex*R). Target genes, oligonucleotide primer sequences and the expected product size in different PCR assays are listed in Table 1. Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A generuler 100 bp ladder (Fermentas, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

RESULTS AND DISCUSSION

Phenotypic characterization of *Pseudomonas* spp.

Thirty isolates from nasal swab samples of 250 camels with the percentage (12%) as shown in Table 2 were produced characteristic bright green color growth features

of *Pseudomonas* species on the cetrimide agar medium. All isolates were reinvestigated biochemically by GN card of Vitek 2 system (bioMe´rieux) and all isolate were confirmed *as Pseudomonas aeruginosa* as automated biochemical tests for *P. aeruginosa*.

Phenotypic detection of ESBL by double disk synergy test method (DDST)

Phenotypic detection of ESBL by DDST revealed that 17 *P. aeruginosa* were ESBL producing *P. aeruginosa* isolates. So, a total percentage of ESBL was detected in a percentage of 56.6% (17/30) from nasal swab isolates.

Antimicrobial susceptibility testing

The results of the antimicrobial susceptibility testing for the 17 ESBL *P. aeruginosa* shows a high-level resistance (100%) to3rd generation cefotaxime, 4th generation cefepime, followed by carbapenem: Meropenem and imipenem (88.2%) and (82.3%) and penicillin (82.3%), gentamicin (76.4%), aztreonam (70.5%), erythromycin (29.5%), sulphamethoxazole/trimethoprim (29.5%), and highly sensitive for ofloxacin (100% sensitive) as shown in Table 3.

Table 1: Primer names, target genes, oligonucleotide primer sequences and the expected product size used in different PCR assay.

Gene	Primer sequence 5'-3'	Amplified product (bp)	Reference
P. aeruginosa 16S rDNA	GGGGGATCTTCGGACCTCA	956	Spilker *et al.,* 2004
	TCCTTAGAGTGCCCACCCG		
*exo*U	CCGTTGTGGTGCCGTTGAAG	134	Winstanley *et al.*, 2005
	CCAGATGTTCACCGACTCGC		
pslA	TCCCTACCTCAGCAGCAAGC	656	Ghadaksaz *et al.*, 2015
	TGTTGTAGCCGTAGCGTTTCTG		
*tox*A	TGTTGTAGCCGTAGCGTTTCTG	396	Matar *et al.*, 2002
	CGCTGGCCCATTCGCTCCAGCGCT		
blaTEM	ATCAGCAATAAACCAGC	516	Colom *et al.*, 2003
	CCCCGAAGAACGTTTTC		
blaSHV	AGGATTGACTGCCTTTTTG	392	
	ATTTGCTGATTTCGCTCG		
blaCTX-M	ATG TGC AGY ACC AGT AAR GTK ATG GC	593	Archambault *et al*, 2006
	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		
mexR	GCGCCATGGCCCATATTCAG	637	Sánchez *et al.*, 2002
	GGCATTCGCCAGTAAGCGG		

Table 2: No. and % of *P. aeruginosa* isolated form camels' nasal swab.

Samples	Number of samples	Number of positive isolates	Percentage
Nasal swab from apparent healthy camels	150	8	5.3%
Nasal swab from diseased camels with respiratory manifestations	100	22	22.0%
Total	250	30	12%

Table 3: Antibiotic resistance pattern of 17 ESBL *P. aeruginosa* isolates.

Antibiotic class	Antibiotic	Sensitive		Resistance	
		No.	(%)	No.	%
Quinolones	Ofloxacin (5 µg)	17	100%	0	0%
Aminoglycosides	Gentamicin (10 µg)	4	23.5%	13	76.4%
Sulfonamides	Sulphamethoxazole/trimethoprim (25 µg)	12	70.5%	5	29.5%
4th generation cephalosporin	Cefepime (30 µg)	0	0%	17	100%
3rd generation cephalosporin	Cefotaxime (30 µg)	0	0%	17	100%
β-lactamase	Penicillin G (10µg)	3	17.6%	14	82.3%
	Aztreonam (30 µg)	5	29.4%	12	70.5%
Carbapenems	Imipenem (10 µg)	3	17.6%	14	82.4%
	Meropenem (1.0 µg)	2	11.7%	15	88.2%
Macrolides	Erythromycin (15 µg)	12	70.5%	5	29.5%

Table 4: Virulence genes, ESBL encoding genes, Multidrug resistance gene profile of *P. aeruginosa* isolates.

Genes / isolate no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Total +ve	%
P. aeruginosa 16S rDNA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	17	100%
*psl*A	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	-	+	5/17	29.4%
*tox*A	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	4/17	23.5%
*exo*U	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	3/17	17.6%
*bla*TEM	+	+	-	+	+	+	+	+	-	+	-	-	+	-	-	+	+	11/17	64.7%
*bla*SHV	+	-	+	+	-	-	-	+	-	-	+	-	-	-	+	+	+	8/17	47.0%
*bla*CTX-M	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	5/17	29.4%
*mex*R	+	+	+	+	+	+	-	+	-	-	-	-	+	-	-	+	+	10/17	58.8%

Molecular detection of ESBL encoding genes, virulence genes, multidrug resistance gene

As shown in Table 4, all 17 ESBL (100%) isolates had confirmed to 16S rDNA gene (Figure 1). PCR screening of genes encoding ESBL revealed the amplification of bla_{TEM}, bla_{SHV} and bla_{CTXM} genes in tested isolates as follow eleven out of 17 ESBL-positive isolates had bla_{TEM} (64.7%), eight had bla_{SHV} gene (47.0%) and five carried bla_{CTX-M} gene (29.4%) as shown in (Figures 3, 4, 5, respectively). According to virulence genes profile of *tox*A, *exo*U and *psl*A, the results revealed that *pslA* gene presented in a percentage of (5/17) 29.4%, *tox*A found in an incidence of (4/17) 23.5% and *exo*U (3/17)17.6% as shown in (Figures 6, 7, 8 respectively). Ten out of 17 *P. aeruginosa* harbored multidrug resistance gene *mex*R in percentage 58.8%.as shown in Figure 2.

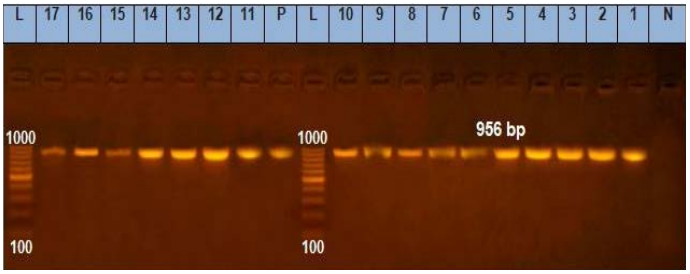

Figure 1: PCR for *P. aeruginosa16S rDNA* (956bp). Lane L: 100-1000 bp ladder molecular size (DNA marker). Lane P.: Control positive *P. aeruginosa*. Lane N.: Control negative. Lanes from 1 to 17: Positive *P. aeruginosa 16S rDNA* (956bp).

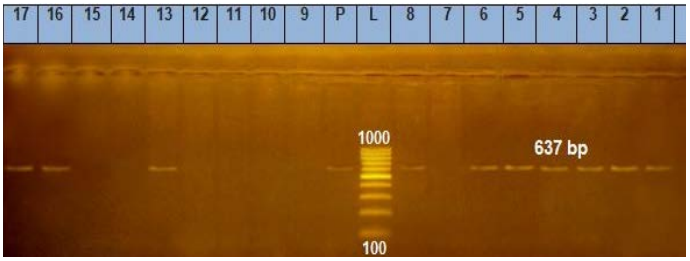

Figure 2: PCR for *mex*R *gene at* (637pbb). Lane L: 100-1000 bp ladder molecular size (DNA marker). Lane P.: Control positive. Lane N.: Control negative. Lanes 1to6,8,13,16,17: Positive for *mex*R (637bp).

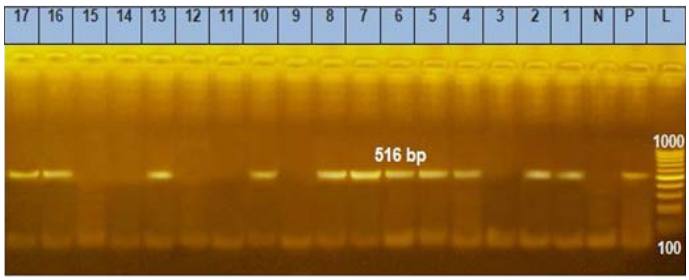

Figure 3: PCR for *bla*TEM *gene at (516bp)*. Lane L: 100-1000 bp ladder molecular size (DNA marker). Lane P.: Control positive *P. aeruginosa*. Lane N.: Control negative. Lanes 1, 2, 4 to 8, 10, 13, 16, 17: Positive for *bla*TEM gene (516pb).

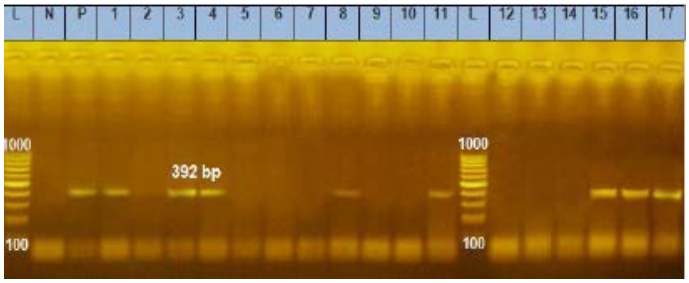

Figure 4: PCR for *bla*SHV gene at (392bp). Lane L: 100-1000 bp ladder molecular size (DNA marker). Lane P.: Control positive. Lane N.: Control negative. Lanes 1, 3, 4, 8, 11, 15, 16, 17: Positive for *bla*SHV gene(392bp).

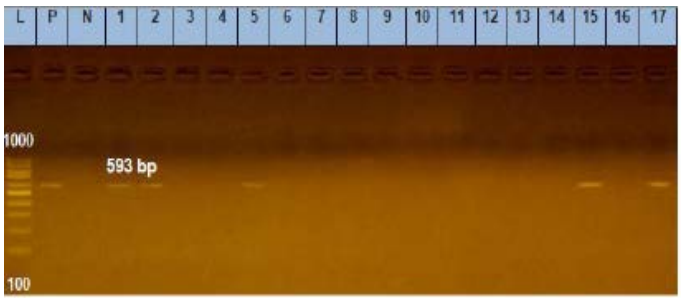

Figure 5: PCR for *blaCTX-M gene at (593bp)*. Lane L: 100-1000 bp ladder molecular size (DNA marker). Lane P.: Control positive. Lane N.: Control negative. Lanes 1, 2, 5, 15, 17: Positive for *blaCTX-M gene (593pb)*.

The dromedary camel is a good source of meat and milk in semiarid and arid zones. This is because of the unique

physiological characteristics of camels (Kadim et al., 2008) Therefore, as they are important food sources, the camel industry is in transition from nomadism to intensive production (FAO, 2019).

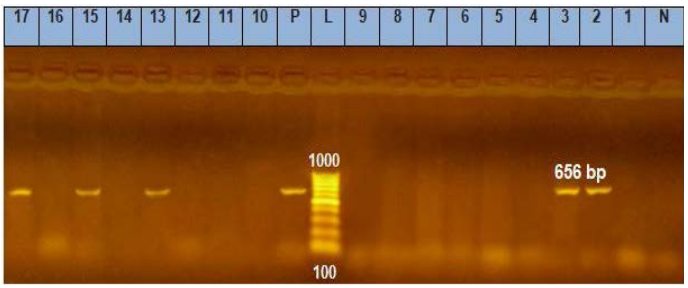

Figure 6: PCR for *pslA gene at (656bp).* Lane L: 100-1000 bp ladder molecular size (DNA marker). Lane P.: Control positive. Lane N.: Control negative. Lanes 2, 3, 13, 15, 17: Positive for *pslA gene (656bp).*

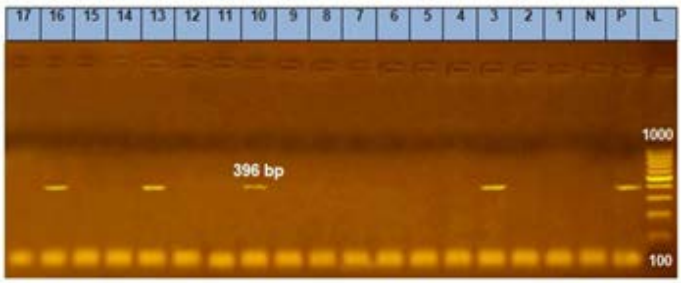

Figure 7: PCR for *toxA* gene at (396bp). Lane L: 100-1000 bp ladder molecular size (DNA marker). Lane P.: Control positive. Lane N.: Control negative. Lanes 3, 10, 13, 16: Positive for *toxA* gene (396bp).

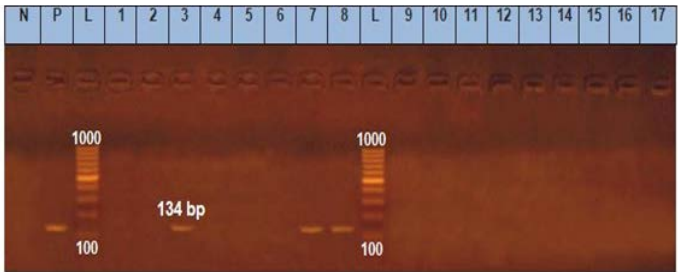

Figure 8: PCR for *exoU* gene at (134bp). Lane L: 100-1000 bp ladder molecular size (DNA marker). Lane P.: Control positive. Lane N.: Control negative. Lanes: 3, 7, 8: Positive for *exoU* gene (134bp).

Pseudomonas aeruginosa is one of the major causes of diseases in camel such as hemorrhagic pneumonia, otitis, mastitis, endometritis, and urinary tract infections (Salomonsen, 2013). In this study, the prevalence of *P. aeruginosa* in camels was determined about 12.0% (30/250). This percent is so nearly to the encountered investigations from camel respiratory tract in Egypt 11.0% which considered as one of the most common opportunistic Gram-negative bacteria (Ismail et al., 2014). *Pseudomonas aeruginosa* and other gram-negative bacteria with ESBLs contain other β-lactamases that makes difficult the phenotypic detection of ESBL (Manchanda and Singh, 2003). All of ruminants depend on eructation, which directly reflected on the microflora in the nasal passages and that explain the main reason for highest percent of bacterial isolation form nasal swabs either in diseased or apparent healthy camels that shown in Table 2. The authors attributed the little increase in result to that, most of samples are collected from animals at regions of pre slaughtering or after transportation, which the animals may subjected to various stress and predisposing factors could augment *P. aeruginosa* growth and increase it recovery rate from respiratory passages. *P. aeruginosa* which considered as one of the most common opportunistic Gram-negative bacteria (Tavajjohi et al., 2011) In this point, there is a considerable report for potential transfer of *P. aeruginosa* in between animal and humans.

Food-producing animals may be an important vehicle for the community wide dissemination of antimicrobial resistant *Enterobacteriaceae* and *P. aeruginosa* especially ESBL-producing type isolates have been found in increasing numbers in food-producing animals (Zurfluh et al., 2016).

ESBL-producing bacteria are one of the fastest emerging resistance problems worldwide. ESBL-producing bacteria were observed in human medical practice, the observation of these bacteria in companion animals and the increase in livestock has initiated monitoring studies concentrating on livestock (Ewers et al., 2011). Accordingly to the hypothesis that animals might become infection sources or even natural persistent sources acting as risky reservoirs of infection leading to the spread of these bacteria specifically multidrug resistant types in community (Watkins and Bonomo, 2016). Molecular identification clearly indicated the presence of virulence gene among studied isolates. *psl*A was present in 29.4% of examined isolates, while *tox*A 23.5% and exoU 17.6% as shown in Table 4 the *tox*A gene represented in percentage (35.29%) in Fazeli and Momtaz (2014) while (Azimi et al., 2016) mentioned that 52% of the isolates carried exoU, and 26.3% carried exoS.

ESBLs are typically identified in *P. aeruginosa* isolates and showing resistance to the extended-spectrum cephalosporin (ESCs) (Fadlelmula et al., 2016), this resistance is often due to the production of β-lactamases. Clinically, ESBLs are generally encoded by plasmid-mediated bla genes; three major clinically relevant β-lactamase genes are *bla*SHV, *bla*TEM *and bla*CTX-M (Bush, 2013). The total percentage of ESBL producing *P. aeruginosa* was 56.6% (17/30) from camel samples by DDST, accordingly, the most frequently β-lactamase-genes detected in this isolate by PCR using specific primers were *bla*TEM (64.7%) followed by *bla*SHV (47.0%) and *bla*CTX-M (29.4%).

The ESBL encoding genes also detected from camels' meat samples in Egypt as follow *bla*CTX-M (38%), followed by *bla*SHV (33.3%) and *bla*TEM, *bal*PER-1 (28.5%) (Elhariri et al., 2017) this indicates the variation of ESBL encoding genes from different isolates which prove that *P. aeruginosa* and other gram-negative bacteria with ESBLs contain other β-lactamases that makes difficult the phenotypic detection of ESBL (Chander and Raza, 2013) this issue need further investigation so, in this study by using PCR for identify *mex*R gene which present in percentage 58.8% (10/17) from *P. aeruginosa* isolates. Bacterial multidrug efflux pumps play an important role in the antimicrobial resistance of gram-negative pathogens (Poole, 2001). In the present study Antibiotic resistance pattern of ESBL producing *P. aeruginosa* showed high-level resistance (100%) to 3rd generation cephalosporine cefotaxime and 4th generation cephalosporine cefepime followed by meropenem (88.2%) and imipenem (82.4%) and Penicillin G, Gentamicin and aztreonam (82.3%, 76.4% and 70.5%), respectively with high sensitive for ofloxacin (100%) followed by Sulphamethoxazole/ trimethoprim and erythromycin (70.5%) as shown in Table 4. This pattern are nearly similar to ESBL *P. aeruginosa* which high level resistance (100%) to ceftazidime, ceftriaxone and rifampicin followed by cefepime (95.2%) and aztreonam (76.1%) (Elhariri et al., 2017) and *P. aeruginosa* are multi-drug resistant to amikacin (17.25%), ciprofloxacin (27.59%), ceftriaxone varied from 51.0 to 73.0% and all the strains were susceptible to imipenem (20.69%) (Chander and Raza, 2013). So, the presence of high resistance profile by camel *P. aeruginosa* isolates my attributed antibiotics used in management of this animals or natural resistance of camel that suites it as a risk reservoir for such pathogens.

CONCLUSIONS AND RECOMMENDATIONS

P. aeruginosa is an important incriminated pathogen in camel. Increasing resistance to beta-lactams in *P. aeruginosa* has become a serious threat, particularly against third and fourth generation cephalosporins. There are a lot of molecular mechanisms to develop resistance against these antibiotics; generation of extended-spectrum beta-lactamases (ESBL), by incorporation of *bla* genes in integrons and inability of porin genes to enhance their expression level and/or alteration of antibiotic target sites.

NOVELTY STATEMENT

As shortage of papers on camel diseases, this paper shed light on the Pseudomonas and their importance in camels as has become a serious threat as well as detection of their virulence genes particularly resistance development against 3rd and 4th generation cephalosporins.

AUTHOR'S CONTRIBUTION

All authors share in the work design, practical section as well as, analysis of the results, writing and revising of the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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