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Antibiotic Susceptibility and Molecular Detection of Virulent *Pseudomonas aeruginosa* Isolated from Bovine Mastitis Milk in Egypt

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the most common causative agents causing mastitis in dairy cattle, which is primarily responsible for the dairy farms' significant economic losses. The purpose of this study was to investigate the prevalence, antimicrobial sensitivity profiling, phenotypic detection of extended-spectrum ß-lactamase (ESBL) producing isolates, molecular identification of major virulence factors (*toxA*, *lasB*, and *exoS*), and biofilm profiles of *P. aeruginosa* strains isolated from mastitis milk. A total of 200 mastitic milk samples were collected from cows and subjected to bacteriological examination. Suspected colonies were confirmed by PCR targeting the 16S rRNA gene. Positive isolates were genotypically examined to determine the presence of virulence factors (*toxA*, *lasB*, and *exoS*). An antimicrobial susceptibility test was carried out on all positive isolates. The phenotypic detection of ESBL was done using the combination disc test (CDT) and the double disc synergy test (DDST). Finally, biofilm production was accessed through the tube adherent method (TA). Among the total samples, *P. aeruginosa* was identified in 15% (n = 30) of the retrieved isolates. Virulence genes (*toxA*, *exoS*, and *lasB*) were detected in 100%, 83.3%, and 66.6% of isolates, respectively. Antimicrobial susceptibility testing showed high sensitivity to imipenem, meropenem, ciprofloxacin, colistin, and gentamicin, while all isolates were completely resistant to amoxicillin, penicillin, ceftazidime, ceftriaxone, streptomycin, erythromycin, doxycycline, and spectinomycin. CDT confirmed ESBL production in 15 isolates (50%) and DDST confirmed it in 10 isolates (33.3%). Biofilm formation by the TA method revealed that 33.3% were strong, 33.3% were moderate, 16.6% were weak, and 16.6% were nonadherent isolates. In conclusion, *P. aeruginosa* strains on dairy farms that harbor virulence genes, produce biofilm, and are resistant to the most popular antimicrobial drug can be hazardous not only to the dairy industry but also for public health.

KEYWORDS
Pseudomonas aeruginosa, Mastitis milk, ESBLs, Antibiotic susceptibility, Virulence genes, Biofilm formation.

INTRODUCTION

Mastitis is a serious problem among dairy farms because of the significant economic loss as well as the negative impacts on animal health and milk quality. *P. aeruginosa* is one of the most common pathogenic etiological agents of bovine mastitis recovered from mastitic cows (Rawat *et al*., 2021). *P. aeruginosa* mastitis increases the number of somatic cells in milk (SCCs) while decreasing major milk components like fat, calcium, lactose, and casein, resulting in severe financial loss within the dairy farm industry (Park *et al*., 2014). Because it thrives in a wide variety of environments, it can affect cattle via the teat canal (Park *et al*., 2014). The disease usually causes inflammation of the mammary glands and can sometimes progress to necrosis and gangrene in the glands. Mastitis is frequently followed by immune system weakness, which can occur because of stress or malnutrition. Additionally, nipple injuries seem to be a significant risk factor (Mahmoudi *et al*., 2019).

P. aeruginosa employs a diverse set of toxin elements, particularly enzymes that can generate cytotoxicity and inducing tissue disorders (Wu *et al*., 2015). Exotoxin A, exoenzymes (ExoU, ExoS, ExoY, and ExoT), and elastase (LasB) are among the virulence components carried by the bacteria, which cause inflammatory reactions and cell death (Park *et al*., 2014). Exotoxin A is the major toxin of *P. aeruginosa*, and it is released from the cell by the type II secretory system. It has a crucial role in *P. aeruginosa* virulence and pathogenicity as it inhibits protein synthesis and causes cell death in the same way as diphtheria toxin. During infection, ExoA is believed to inhibit the host immune response. Additionally, it was demonstrated to have a significant impact on local tissue injury as well as the invasion process (Urgancı *et al*., 2022).

P. aeruginosa secretes a variety of proteases, including elastase and alkaline protease, which have been associated with pathogenicity. Proteases have tissue-damaging ability and can degrade a variety of plasma proteins, coagulation factors, and complement factors (Peters and Galloway, 1990). The *lasB* gene is a metalloprotease capable of breaking down fibrin, elastin, mucins, tissue collagen and damaging the epithelium of the respiratory tract, which helps in *P. aeruginosa* invasion and persistence in tissues (Hoge *et al*., 2010).

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ExoS, a toxic effector protein, enters host cells via the type III secretory systems (TTSS), which contributes to epithelial surface disruption and cell death. ExoS is also implicated in bacterial cell attachment, phagocytosis, systemic dissemination, and immunity evasion, resulting in elevated and increased mortality rates (Vance *et al*., 2005; Le Berre *et al*., 2011).

Antimicrobial resistance is defined generally as an organism's ability to resist the action of an antimicrobial agent to which it was previously sensitive (Bagge *et al*., 2004). *P. aeruginosa* is one of the most common serious pathogens implicated in antibiotic resistance (Azam and Khan, 2019). It displays intrinsic resistance to several antimicrobial drugs, including a low-permeability outer membrane (1/100 of the permeability of *E. coli*'s outer membrane); other mechanisms, such as the efflux system, which expel antibiotics out of the bacterial cell, and the synthesis of antibiotic-inactivating enzymes, are also responsible for their intrinsic resistance. *P. aeruginosa* strains can develop acquired resistance involving the acquisition of a resistance gene via integrons or plasmids or a mutation in genes expressing porins, efflux pumps, penicillin-binding proteins, and chromosomal β -lactamase, all of which contribute to resistance to ß-lactams, carbapenems, aminoglycosides, and fluoroquinolones (Pachori *et al*., 2019).

Extended-spectrum β-lactamase enzymes (ESBLs) represent the most common β-lactamases identified with bacteria belonging to the Enterobacteriaceae as well as other Gram-negative pathogens. ESBL enzymes are well known for their ability to hydrolyze cephalosporin (both third and fourth generation) and monobactams, but not hydrolyze carbapenems (meropenem and imipenem). Penicillin-lactamase inhibitor combinations such as ampicillin with sulbactam combination or amoxicillin with clavulanic acid represent the most used drugs in the veterinary field for bovine and domesticated animals because they have minimal adverse side effects (Pehlivanoglu and Sababoglu, 2021).

P. aeruginosa, like other bacteria, generates a variety of biofilms based on its environment (Gupta and Schuster, 2012). Biofilms are tightly packed populations of microorganisms that surround themselves with extracellular matrix (Gulati and Nobile, 2016). The extracellular matrix (ECM) is the hallmark of biofilms and is composed of proteins, polysaccharides, nucleic acids, and lipids. It can serve as a structural scaffold as well as a protective barrier (Koo and Yamada, 2016). The majority of *P. aeruginosa* strains recovered from mastitic cows are powerful biofilm producers, which can impair antibiotic efficiency and contribute to chronic mastitis (Melchior *et al*., 2006). Biofilm is thought to be the hot spot for the accumulation and transmission of antibiotic resistance genes (ARGs) (Balcázar *et al*., 2015). Therefore, this study was carried out to explore the prevalence, virulence-associated genes, antibiotic susceptibility phenotypes, β-lactamase resistant (ESBls) isolates, and biofilm profiling of *P. aeruginosa* isolates obtained from mastitis milk.

MATERIALS AND METHODS

Ethical approval

Animal handling and collection of samples were followed by animal ethics committee guidelines, Faculty of Veterinary Medicine, Mansoura University, Egypt. (Protocol code: M/104).

Sample Collection

Mastitis milk samples (n. = 200) were collected from various dairy farms in Dakahlia Governorate, Mansoura, Egypt. From March 2021 to November 2021, samples were collected on a regular basis from ten different farms. In sterile cups, milk samples were obtained aseptically and directly from the udders of mastitis cows showing clinical signs of mastitis, such as swollen udder quarters or entirely swollen udders, and abnormal milk secretion (flakes or clots). To reduce contamination and maximize the chances of obtaining relevant information from the milk culturing procedure, the following protocols for the aseptic collection of clean milk samples were followed: Wearing gloves, withdraw three or four streams of milk from the quarter being sampled to reduce sample contamination. Brush any dirt, dust, or bedding particles from the udder and teats. Following removal, predip the teat with an effective teat dip (0.5% iodine) for at least 20 to 30 seconds and use a dry paper towel to remove the predip from each teat. Scrub the teat end and orifice with a cotton pad dampened with 70% isopropyl alcohol for 20 seconds. All milk samples were taken under strictly sanitary conditions in closed, labeled sterile cups. Samples were then kept cold and preserved in an insulated tank containing ice bags and transported immediately to the Department of Bacteriology, Mycology, and Immunology, Faculty of Veterinary Medicine, Mansoura University, for bacteriological analysis.

Isolation of P. aeruginosa isolates

Milk samples were inoculated onto Cetrimide and MacConkey's agar (Oxoid, England) and incubated for 24–48 hours at 37°C. *P. aeruginosa* isolates were initially identified depending on the features of the colony on the agar surfaces (pale, non-lactose fermenter on MacConkey's and yellowish green pigment with a fruity odor on Cetrimide agar). A single suspected colony was selected from the cetrimide plate, purified via spreading on Tryptic Soya Agar (TSA) (Oxoid, England), and kept at 4°C for staining by Gram's stain and other biochemical analysis (Su *et al*., 2018). All suspected isolates were kept on 60% glycerol at -70°C for further identification. To confirm viability prior to testing, each isolate was subcultured twice on TSA media for 24 hours at 37°C.

Extraction of bacterial genomic DNA

P. aeruginosa genomic DNA was extracted using the boiling method as described by Ahmed and Dablool (2017). Briefly, a suspension of pure suspected biochemically and morphologically identified colonies from each *P. aeruginosa* isolate was heated at 95°C for 20 minutes using a Biometra heat block, and then all tubes were centrifuged for 10 minutes. The resulting supernatant was placed in a new eppendorf tube and kept at -20°C for subsequent molecular identification.

Molecular identification of P. aeruginosa using conventional PCR targeting 16S rRNA

PCR was performed on all extracted DNA by using species-specific primers targeting 16S rRNA (Metabion, Germany) using the following primer pairs: Forward: GGGGGATCTTCGGACCTCA, and reverse: TCCTTAGAGTGCCCACCCG. The following amplification program, according to Spilker *et al*. (2004), was used: Primary denaturation at 95˚C for 2 min, 25 cycles of secondary denaturation at 95˚C for 2 min, annealing at 58˚C for 1 min, extension at 72˚C for 20 sec, and a final extension at 72˚C for 7 min. PCR was carried out in a 20 µl reaction containing 10 µl of DreamTaq Green PCR Master Mix (Thermo Scientific, USA), 1 µl of each primer at a 20 pmol concentration, 3µl of nuclease-free water, and 5 µl of the template DNA.

The reactions were carried out in a Biosystems 2720 thermal

cycler (Thermo Fisher Scientific, USA). The PCR products of each reaction were separated by electrophoresis in 1% agarose gel at 0.8% (w/v) stained by ethidium bromide in 1x TBE buffer at room temperature. A 100-bp DNA ladder (Qiagen, USA) was used to determine the fragment sizes. A negative control was run for amplification. The gel was visualized by a UV gel documentation device (Cleaver Scientific Ltd., ultra violet gel documentation system,Rugby, Warwickshire, UK). Cyclic PCR conditions, fragment size, and thermal profile are listed in Table 1.

Molecular detection of virulence genes

A uniplex PCR assay was performed to detect the three selected virulence-associated genes: elastase enzyme (*lasB*), exotoxin A (*toxA*), and exoenzyme S (*exoS*), following a protocol described by Wolska and Szweda (2009). Primer sequences, cyclic PCR conditions, and fragment size for each gene are listed according to the referenced author in Table 1. PCR products were separated in a 1% agarose gel stained with ethidium bromide for 1 h at 100 V and detected by UV transillumination. Amplified genes were identified on the basis of fragment size.

Antimicrobial susceptibility testing

The antimicrobial test of *P. aeruginosa* isolates was done using the Kirby–Bauer disc diffusion method against 18 antibiotics (Oxoid, UK) comprising 9 different antimicrobial classes. The culture of each isolate with 0.5 McFarland concentration was distributed onto the surface of Mueller–Hinton agar (Oxoid, UK). Then antibiotic discs of penicillin (P, 10iu), amoxicillin (AX, 10µg), meropenem (MEM, 10µg), imipenem (IPM, 10µg), cefotaxime (CTX, 30µg), cefuroxime (CXM, 30µg), ceftriaxone (CRO, 30µg), ceftazidime (CAZ, 30µg), amikacin (AK, 30µg), gentamicin(CN, 10µg), apramycin (APR, 15µg), streptomycin (S, 10µg), kanamycin (K, 30µg), colistin (CT, 10µg), spectinomycin (SPT, 100µg), erythromycin (E, 15µg), ciprofloxacin (CIP, 5µg), and doxycycline (DO, 30µg) were smeared on the agar surfaces and incubated at 37°C for 24 hours, the zones of inhibition were measured and interpreted according to the Clinical and Laboratory Standard Institute recommendations (CLSI, 2019) as shown in Table 2. MDR was defined as resistance to at least one antimicrobial agent in three or more antimicrobial categories (Saderi and Owlia, 2015). For each isolate, the MDR index (MDRI) was calculated (Table 3) by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics to which the isolate had been exposed (Doughari *et al*., 2011).

Phenotypic detection of ESBL producing isolates

To identify ESBL-producing *P. aeruginosa* isolates, the combination disc test (CDT) using ceftazidime disc (CAZ, 30 g) as well as amoxicillin-clavulanic acid (AMC, 20 g/10 g) was applied. On the agar plates, the CAZ and AMC discs were positioned 15 mm apart. The isolates were recognized as ESBL-positive when an inhibition zone of ≥5 mm around the disc of amoxicillin-clavulanic acid and not around the disc with ceftazidime was produced. The double disc synergy test (DDST) was employed as a further phenotypic screening method to identify ESBL-producing *P. aeruginosa* strains, with discs containing amoxicillin-clavulanic acid as a source of ESBL inhibitors and both (cefotaxime-CTX 30 g & ceftazidime-CAZ 30 g). The AMC disc was placed in the center of the plate, and the two third-generation cephalosporin discs, CAZ and CTX, were placed 15 mm (center to center) distant from the AMC disc. When a clear enlargement of the inhibitory zone towards the discs with amoxicillin-clavulanic acid was detected, the DDST test was considered positive for an ESBL isolate (Laudy *et al*., 2017). The two disc diffusion tests were carried out on Müller-Hinton agar (Oxoid, UK) using a sterile cotton swab and a *P. aeruginosa* isolate culture of 0.5 McFarland concentration. All the plates were incubated overnight at 37°C.

Qualitative detection of biofilm with the tube method

Each isolate was smeared on the surface of trypticase soya agar (TSA; Oxoid, England) and incubated for 24 hours at 37°C. A loopful of organisms was inoculated in a tube containing 5 mL of tryptic soya broth with 1% glucose (TSBG) for enritchment and incubated at 37°C for 18 hours without shaking. After incubation,

Table 1. Oligonucleotide primers, fragment size, and thermal profiles used for molecular identification

Target primer	Primer direction and sequence	Fragment size (bp)	PCR conditions	Reference
16S rRNA	F: GGGGGATCTTCGGACCTCA R: TCCTTAGAGTGCCCACCCG	956	95˚C/ 2 min 25 cycles of 94˚C/ 20 sec, 58˚C/ 20sec, and 72˚C/ 40sec 72˚C/ 7 min	Spilker *et al*. (2004)
lasB	F: GGAATGAACGAAGCGTTCTCCGAC R: TTGGCGTCGACGAACACCTCG	284	94˚C/ 3 min 30 cycles of 94˚C/ 30 sec 57˚C/ 60 sec 72˚C/ 60 sec 72˚C/ 5 min	Wolska and Szweda (2009)
toxA	F: CTGCGCGGGTCTATGTGCC R: GATGCTGGACGGGTCGAG	270	94˚C/ 3 min 30 cycles of 94˚C/ 30 sec 57˚C/ 60 sec 72˚C/ 60 sec 72˚C/ 5 min	Wolska and Szweda (2009)
exoS	F: CGTCGTGTTCAAGCAGATGGTGCTG R: CCGAACCGCTTCACCAGGC	444	94˚C/ 3 min 30 cycles of 94˚C/ 30 sec 57˚C/ 60 sec 72˚C/ 60 sec 72˚C/ 5 min	Wolska and Szweda (2009)

the tubes were gently aspirated and washed with phosphate-buffered solution (PBS, pH 7.4). The tubes were then inverted until completely dry (30 minutes), then dyed for 15 minutes using 1% crystal violet, rinsed with deionized water,and left to dry in an inverted position. In addition, there was also a negative control tube with only TSBG but no bacterial growth. Each isolate was tested in triplicate. The production of biofilms was observed with the naked eye. Positive outcomes were seen as a violet color on the bottom and walls of the tubes. The biofilm-forming capacity of each strain was categorized as strongly adherent, moderately adherent, weakly adherent, or nonadherent according to the strength of the violet color (Hukić *et al*., 2018).

RESULTS

Prevalence and molecular characterization of P. aeruginosa isolates

Out of the investigated mastitic milk samples (n = 200), 30 *P. aeruginosa* was biochemically detected. The isolates were Gram-negative rods that produced a bluish-green pigment on cetrimide agar plates and a colorless colony with no lactose fermentation on MacConkey's agar plates (Fig. 1). All isolated strains were catalase and oxidase positive. By microscopic examination, suspected isolates were Gram-negative rods by Gram staining (Fig. 2). Further confirmation was performed on the biochemically identified *P. aeruginosa* using a PCR assay targeting the 16S rRNA gene. The expected 956-bp product size was successfully amplified in all the tested isolates with a prevalence of 15% (30/200) (Fig. 3).

Virulence gene profiles

Positive *P. aeruginosa* strains were examined to determine the existence of virulence genes. The PCR assay revealed that *toxA* represented the most abundant toxin gene discovered amongst the tested strains (30/30; 100%), followed by the *exoS* gene (25/30; 83.3%) and the *lasB* gene (20/30; 66.6%) (Fig. 4).

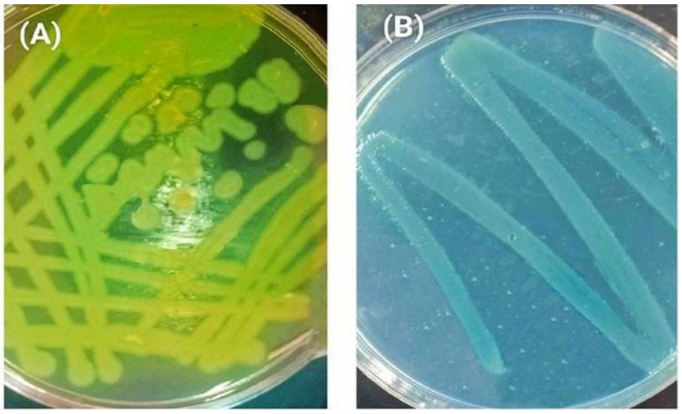

Fig. 1. *P. aeruginosa* on cetrimide agar plate. A: showed pyoviridine pigment B: pyocyanine pigment.

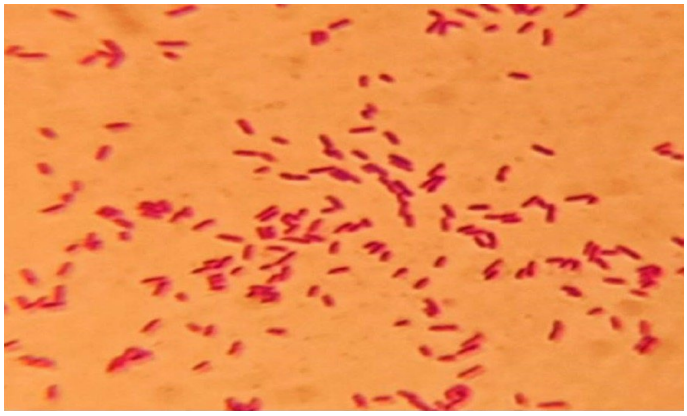

Fig. 2. Gram-negative rod-shape of *P. aeruginosa* under light microscope.

Table 2. Percentage of antimicrobial susceptibility for *P. aeruginosa* isolates.

Antimicrobial agent	Family	Disc code	CPD	*P. aeruginosa*					
				Resistance		Intermediate		Sensitive	
				No	%	No	%	No	%
Penicillin	β-lactams	P	10 U	30	100	0	0	0	0
Amoxicillin		AX	10µg	30	100				
Imipenem	Carbapenems	IPM	10µg	0	0	9	30	21	70
Meropenem		MEM	10µg	0	0	5	16.6	25	83.3
Ceftriaxone	Cephalosporin	CRO	30µg	30	100	0	0	0	0
Cefuroxime		CXM	30µg	17	56.6	7	23.3	6	20
Cefotaxime		CTX	30µg	3	10	27	90	0	0
Ceftazidime		CAZ	30µg	30	100	0	0	0	0
Gentamicin	Aminoglycoside	CN	10µg	11	36.6	5	16.6	14	46.6
Streptomycin		S	10µg	30	100	0	0	0	0
Amikacin		AK	30µg	10	33.3	11	36.6	9	30
Kanamycin		K	30µg	20	66.6	6	20	4	13.3
Apramycin		APR	15µg	11	36.6	15	50	4	13.3
Colistin sulphate	Polypeptides	CT	10µg	10	33.3	0	0	20	66.66
Erythromycin	Macrolides	E	15µg	30	100	0	0	0	0
Spectinomycin	Aminocyclitol	SPT	100µg	30	100	0	0	0	0
Ciprofloxacin	Fluoroquinolone	CIP	5µg	4	13.3	10	33.3	16	53.3
Doxycycline	Tetracycline	DO	30µg	30	100	0	0	0	0

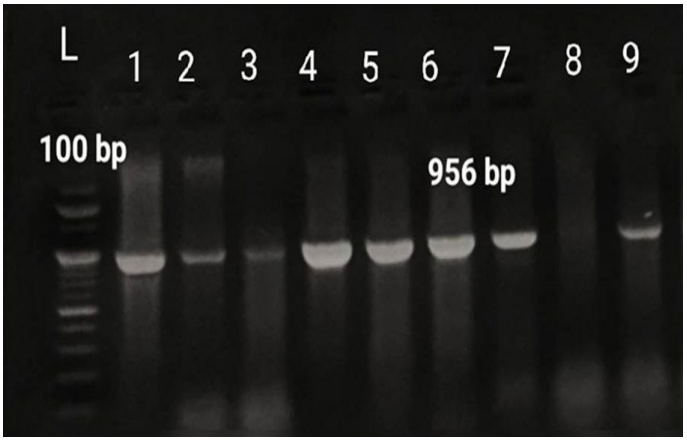

Fig. 3. Agarose gel electrophoresis showing amplification of 16S rRNA gene of *P. aeruginosa* strains. L: 100bp DNA ladder.

Antimicrobial resistance and multi-drug resistance index (MDRI)

The results of antimicrobial susceptibility testing of *P. aeruginosa* positive isolates (n = 30) showed that all isolates were completely resistant (100%) to penicillin, amoxicillin, ceftriaxone, ceftazidime, streptomycin, erythromycin, spectinomycin, and doxycycline, followed by kanamycin (66.6%) and cefuroxime (56.6%), and they displayed an intermediate resistance to cefotaxime (90%), apramycin (50%), and amikacin (36.6%). While *P. aeruginosa* isolates were more sensitive to meropenem (83.3%), imipenem (70%), colistin sulfate (66.66%), ciprofloxacin (53.3%), and gentamicin (46.6%), Table 2. MDR was found in 100% of the isolated strains. The patterns P, AX, CRO, CAZ, S, E, SPT, DO, CXM, and K represented the most common antimicrobial resistance profile found in *P. aeruginosa* isolates. The higher MDR index (0.8) was noticed among 2 isolates that were resistant to 14 types of the 18 tested antibiotic discs. The multidrug resistance index for MDR *P. aeruginosa* isolates ranged from 0.5 to 0.8 (Table 3).

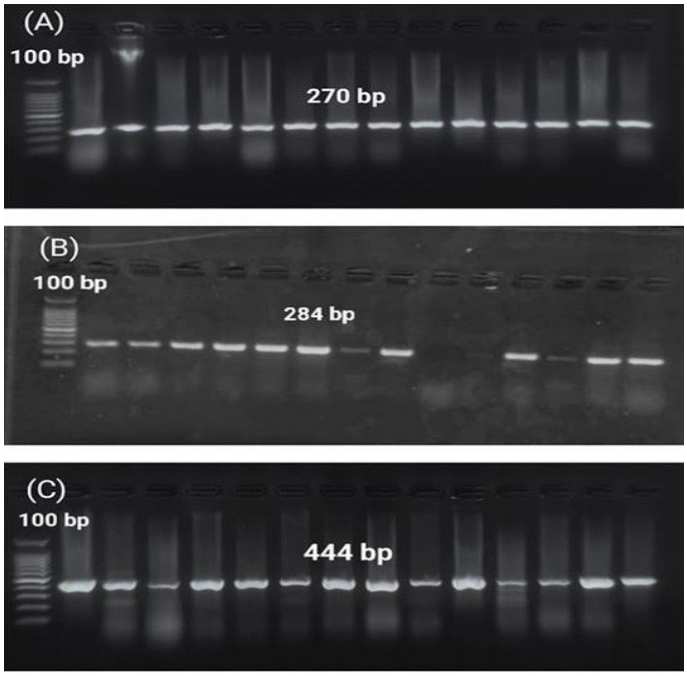

Fig. 4. Agarose gel electrophoresis showing amplification of virulence-associated genes. A: 270bp fragment of *toxA* gene. B: 284bp fragment of *lasB* gene. C: 444bp fragment of *exoS* gene of P.aeruginosa isolates.

Phenotypic detection of ESBL-producing P. aeruginosa isolates

Out of 30 isolates tested for ESBL production, the combination disc test (CDT) could identify 15 (50%) as ESBL producer isolates. The isolates showed a significant zone enhancement with

Table 3. Antimicrobial resistance patterns and antibiotypes of *P. aeruginosa* isolates.

Antibiotypes	Resistance pattern	Isolates no (%)	No. of resistant Antibiotic	No. of resistant antibiotic categories	MDR INDEX
I	P, AX, CRO, CAZ, S, E, SPT, DO, K	2	9/18	6/9	0.5
II	P, AX, CRO, CAZ, S, E, SPT, DO, CXM	3	9/18	6/9	0.5
III	P, AX, CRO, CAZ, S, E, SPT, DO, CT	1	9/18	7/9	0.5
IV	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, K	4	10/18	6/9	0.6
V	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, CT	2	10/18	7/9	0.6
VI	P, AX, CRO, CAZ, S, E, SPT, DO, AK, K	1	10/18	6/9	0.6
VII	P, AX, CRO, CAZ, S, E, SPT, DO, APR, CT	1	10/18	7/9	0.6
VIII	P, AX, CRO, CAZ, S, E, SPT, DO, K, APR	1	10/18	6/9	0.6
IX	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, CTX, K	1	11/18	6/9	0.6
X	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, CN, K	1	11/18	6/9	0.6
XI	P, AX, CRO, CAZ, S, E, SPT, DO, AK, K, APR	1	11/18	6/9	0.6
XII	P, AX, CRO, CAZ, S, E, SPT, DO, CN, AK, APR	1	11/18	6/9	0.6
XIII	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, APR, CT, CIP	1	12/18	8/9	0.7
XIV	P, AX, CRO, CAZ, S, E, SPT, DO, CN, AK, K, APR	2	12/18	6/9	0.7
XV	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, APR, CT, CIP	1	12/18	8/9	0.7
XVI	P, AX, CRO, CAZ, S, E, SPT, DO, CN, AK, K, APR	1	12/18	6/9	0.7
XVII	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, K, CT, CIP	1	12/18	8/9	0.7
XVIII	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, CN, AK, K	1	12/18	6/9	0.7
XIX	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, CN, K, CT, CIP	1	12/18	8/9	0.7
XX	P, AX, CRO, CAZ, S, E, SPT, DO, CN, AK, K, APR, CT	1	13/18	7/9	0.7
XXI	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, CTX, CN, AK, K, APR	1	14/18	6/9	0.8
XXII	P, AX, CRO, CAZ, S, E, SPT, DO, CXM, CTX, CN, AK, K, CT	1	14/18	7/9	0.8

the amoxicillin-clavulanic acid disc (≥5 mm) when compared with ceftazidime discs. While ESBL production by DDST was confirmed in 10 (33.3%) isolates, Positive isolates showed a zone of inhibition toward one or two tested discs (ceftazidime-CAZ 30µg, cefotaxime-CTX 30µg) toward amoxicillin-clavulanic acid (Fig. 5).

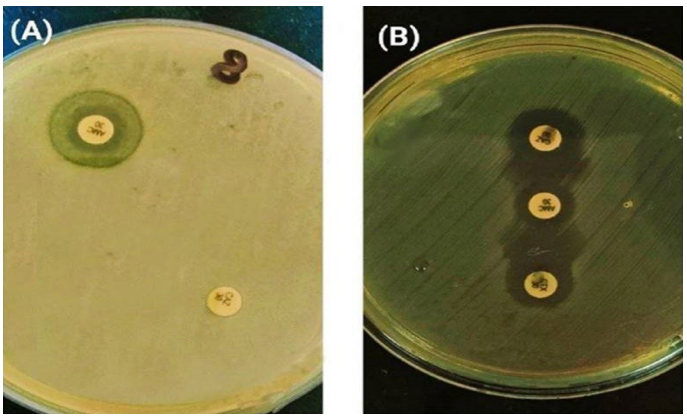

Fig. 5. A): Combination disc test (CDT) for ESBL production confirmed by an increase in zone diameter of ≥5mm toward amoxicillin-clavulanic acid (AMC). B): Double disk synergy test (DDST). Showing enhancement of the zone of inhibition of the two drug disks, ceftazidime (CAZ) 30 µg, and cefotaxime (CTX) 30 µg toward amoxicillin-clavulanic acid.

Biofilm formation

P. aeruginosa isolates (n = 30) were classified using the tube adherent test (TA) according to their ability to produce biofilms as follows: 10 (33.3%) isolates were strongly adherent; 10 (33.3%) isolates were moderately adherent; 5 (16.6%) isolates were weakly adherent; and 5 (16.6%) isolates were non-adherent.

DISCUSSION

P. aeruginosa is a particularly important pathogen in veterinary medicine because it produces a variety of clinical manifestations in livestock and domestic animals. *P. aeruginosa* mastitis in ruminants can happen sporadically or as an outbreak in dairy cows. The infections in the mammary gland can originate from either a single point source or through ongoing exposure to a common contaminant, such as a dirty or polluted environment (Schauer *et al*., 2021). *Pseudomonas* infections in bovine mastitis represent a greater management problem because of their prolonged survival and tendency to disseminate across the herds (Rivera Aguayo *et al*., 2020). One single strain of *P. aeruginosa* may be responsible for the whole outbreak at the dairy farm, so the farmer is instructed to change udder-preparing routines since the transmission mostly happened through a contaminated teat cleaning solution and to cull diseased cows (Schauer *et al*., 2021).

In the present study, out of 200 collected milk samples, 30 *P. aeruginosa* isolates (15%) were recovered and confirmed by PCR targeting 16S rRNA. This result is in line with other studies performed in Egypt (Ghazy *et al*., 2015) and Iraq (Saleh *et al*., 2016) that reported a similar frequency in *P. aeruginosa* strains from bovine mastitis with percentages around 10% and 11.45%, respectively. While higher isolation rates from the mastitis milk of cows (44%, 26.6%, 34%, and 45%) were recorded by Naser (2016); Neamah (2017); Ibrahim *et al*. (2017) and Schauer *et al*. (2021), respectively. On the other hand, a lower prevalence of *P. aeruginosa* from milk samples (5.4%, 6%, 0.61%, 5.15%) has been reported by Banerjee *et al*. (2017); AL-Taee *et al*. (2019); Yadav *et al*. (2020) and Rawat *et al*. (2021), respectively. The differential reporting of *P. aeruginosa* prevalence may be attributed to changes in location, hygiene measures, and farm management practices followed.

Interestingly, *P. aeruginosa* has a diverse set of cell-associated and extracellular virulence factors that contribute to its pathogenicity (Jurado-Martín *et al*., 2021). In this study, *toxA*, *exoS*, and *lasB* were detected in *P. aeruginosa* isolates with percentages of 100%, 83.3%, and 66.6%, respectively. Noticeably, *toxA* was found to be considerably more prevalent than *exoS* and *lasB* in *P. aeruginosa* strains. A similar result was reported by Banerjee *et al*. (2017), who reported that the presence of *toxA* (63.2%) was higher than *exoS* (36.8%) in *P. aeruginosa* strains isolated from mastitic milk in India. Also, Neamah (2017) from Iraq detected *toxA* in 100% of mastitic milk, followed by *exoS* (75%). In Korea, Park *et al*. (2014) detected *exoS* genes in 82.7% of *P. aeruginosa* mastitic milk isolates. In Egypt, higher frequencies were obtained by Tartor and El-Naenaeey (2016), who isolated *toxA*, *lasB*, and *exoS* in 100% of *P. aeruginosa* mastitis milk, while lower results were obtained by Younis *et al*. (2015), who reported the precense of *lasB*, *toxA*, and *exoS* virulence genes with percentages of 14.7%, 8.57%, and 17.14% of tested isolates, respectively. The *toxA* and *exoS* genes are the main fatal weapons associated with cattle subclinical mastitis infections (Banerjee *et al*., 2017). The *exoS* gene plays a potentially important role in inflammatory responses and tissue necrosis to a greater degree than predicted. As a result, the vast majority of *P. aeruginosa* strains harboring *exoS* must be regarded as potentially dangerous to dairy cattle (Park *et al*., 2014).

Antimicrobial resistance represents one of a nation's most critical challenges today and is rapidly rising in developing countries. As a result, it is critical to identify *P. aeruginosa* quickly and accurately, as well as assess their sensitivity pattern; this might also avoid unnecessary antibiotic therapy, which may lead to antibiotic-resistant microorganisms (Hamisi *et al*., 2014). *P. aeruginosa* bovine mastitis is difficult to cure as it's resistance to various medications used for treating mastitis, including amoxicillin, ampicillin, first- and second-generation cephalosporins, amoxicillin/clavulanic acid, and trimethoprim (Schauer *et al*., 2021). This limits the use of a particular antibiotic against this organism, resulting in higher mortality rates (Arumugam *et al*., 2018).

In this study, the antimicrobial sensitivity test of *P. aeruginosa* mastitic milk isolates showed complete resistance to amoxicillin, penicillin, ceftazidime, ceftriaxone, streptomycin, erythromycin, spectinomycin, and doxycycline, followed by kanamycin, and cefuroxime. While an intermediate resistant to cefotaxime, apramycin, and amikacin were detected. On the other hand, all *P. aeruginosa* isolates displayed sensitivity to meropenem, imipenem, ciprofloxacine, colistin sulfate, and gentamicin. Our findings are in agreement with the results from other studies in Egypt by Tartor and El-Naenaeey (2016) who reported that *P. aeruginosa* isolates from mastitis milk exhibited resistance to ampicillin, erythromycin, tetracycline, ceftazidime and high sensitivity to gentamicin, imipenem, ciprofloxacin and colistin. Additionally, In Korea, Park *et al*. (2014) revealed that *P. aeruginosa* isolates showed resistance to erythromycin, tetracycline, amikacin, and kanamycin, all of which were permitted for treating cattle mastitis as well as a feed supplement, while resistance to meropenem and ciprofloxacin, which are both not approved for veterinarian usage, was low. In addition, Ohnishi *et al*. (2011) and Schauer *et al*. (2021) revealed that *P. aeruginosa* isolates from mastitis milk exhibited high susceptibilities to ciprofloxacin, imipenem, meropenem, amikacin, and gentamicin. The high sensitivity of the studied isolates to imipenem, meropenem and ciprofloxacin agreed also with Ghazy *et al*. (2015); Saleh *et al*. (2016); Ibrahim *et al*. (2017) and AL-Taee *et al*. (2019) who recorded a higher sensitivity of *P. aeruginosa* mastitis milk isolates to carbapenems. Carbapenems have a broad antibacterial spectrum and play a fundamental role in the treatment of infections caused by MDR *P. aeruginosa* isolates (Tartor and El-Naenaeey, 2016). Schauer *et al*. (2021) reported that ciprofloxacin can be taken as a good choice of treatment of bovine mastitis.

In the current study, all the isolates were multidrug resistant as they resisted at least one member of six different classes. The multidrug resistance index ranges between 0.5 and 0.8. Our result is in agreement with Tartor and El-Naenaeey (2016) in Egypt who obtained high frequency of MDR *P. aeruginosa* isolates from

mastitis milk.

P. aeruginosa develops ß-lactam resistance via both enzymatic and non-enzymatic mechanisms. The enzymatic process involves the production of penicillinase, cephalosporinases, and imipenemase enzymes. Non-enzymatic mechanisms include reduced membrane permeability because of porin loss and active pumping-out mechanisms (Cavallo *et al*., 2000). ESBLs are inhibited by clavulanic acid/sulbactam, so for phenotypic confirmation of ESBL production isolates, the DDST and CDT tests are used, as they use clavulanic acid/sulbactam inhibitors to reverse ESBL resistance (Drieux *et al*., 2008).

In this study, *P. aeruginosa* isolates were phynotypically examined for ESBL production. The results of two tests detected 15 isolates (50%) by combination disc test (CDT) and 10 isolates (33.3%) by double disc synergy test (DDST). This result agrees with Laudy *et al*. (2017), who detected ESBL-producing *P. aeruginosa* isolates by the two tests, while Pehlivanoglu and Sababoglu (2021) reported that the two phenotypic ESBL tests (CDT and DDST) for *P. aeruginosa* isolates from bovine mastitis milk weren't ESBL producers. The World Health Organization declared that Enterobacteriaceae producing ESBLs enzymes are posing a serious hazard to human health because of a rise in clinical case isolation rates. Similarly, the rate of reports about these ESBLs producing bacteria from infected cattle and domesticated animals is growing in the veterinary field (Pehlivanoglu and Sababoglu, 2021).

P. aeruginosa is a typical opportunistic biofilm-forming bacterium. Biofilm is the major factor in pathogenicity as well as antibiotic resistance (Kunwar *et al*., 2021). *P. aeruginosa* biofilm is responsible for the chronic and recurring infectious diseases in animals that impede bacterial infection treatment. Antibiotics are believed to be inefficient against microorganisms that form biofilms because of the inability of drug molecules to permeate into the inner surface of biofilm (Yuan *et al*., 2019). In dairy farms, *P. aeruginosa* contaminates hose nozzles and milking equipment by producing a biofilm that improves adhesion to pipe and hose surfaces and increases resistance to surfactants, phagocytes, and some antibiotics (Kawai *et al*., 2017).

In this study, the ability of *P. aeruginosa* to produce biofilms was evaluated using the tube adherent method; 10 (33.3%) were strongly adherent isolates; 10 (33.3%) were moderately adherent isolates; 5 (16.6%) were weakly adherent isolates; and 5 (16.6%) were non-adherent isolates. These results were similar to those obtained by Rewatkar and Wadher (2013), who could detect biofilm by the tube adherent method in *P. aeruginosa* clinical isolates. Biofilm formation seems to be the primary cause of the majority of recurrent illnesses. Bacteria inside the biofilm can resist antibiotic treatment and induce recurrent infection because the biofilm structure masks bacteria from the action of antibiotics and the immune system. Following unsuccessful treatment due to biofilm protection, approximately 40% of bovine mastitis progresses into asymptomatic or chronic mastitis (Park *et al*., 2014). As a result, alternative treatment options for farm animals are urgently needed in order to limit antibiotic use. Substantial research is required to create a novel biocompatible as well as non-toxic anti-biofilm agent from naturally biological origin (Rivera Aguayo *et al*., 2020).

CONCLUSION

ESBL-producing *P. aeruginosa* isolated from this study is creating serious problems in dairy farms and public health. Its resistance to various antibiotics, expression of virulent factors, biofilm production inside the milking parlor, and poor environmental hygiene result in significant economic losses. As a result, it's really essential to determine the origin of infection or the mechanisms of transmission and apply appropriate prophylactic measures to stop the spread of microorganisms among herds.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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