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Characterization of antibiotic resistance genes in *Pseudomonas aeruginosa* isolates from Egyptian patients

Alaa M. Soliman[1*], Eman A. Hassan[1], Ayman Abdelkareem[2], Mohammed M. S. Farag[2,3]

[1]International Islamic Center for Population Studies and Research Department, Al-Azhar University, Egypt

[2]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Egypt

[3]Biomedical Research Department, Armed Forces College of Medicine (AFCM), Cairo, Egypt

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Corresponding author:

Alaa M. Soliman, Ph. D

E-mail: alaasoliman629@yahoo.com

Mobile: (+2)01222806275

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ABSTRACT

This study investigates the prevalence and antibiotic resistance profiles of bacteria in human semen, highlighting the risk of antimicrobial resistance (AMR) associated with the overuse of antibiotics. One hundred semen samples were collected from participants at the urology department of El-Hussien Hospital. The samples were screened for bacterial contamination, and isolated strains were tested for sensitivity against commonly used antibiotics. The Gram-positive bacteria were identified *Staphylococcus aureus*, *Bacillus subtilis*, and *Enterococcus faecalis* along with Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, and *Neisseria gonorrhoeae*. Notably, *P. aeruginosa* exhibited the highest antibiotic resistance, confirmed by 16S rRNA sequencing (deposited under GeneBank accession number LC455963). Resistance genes blaTEM and blaCTX, known to contribute to extended-spectrum beta-lactamase (ESBL) production, were detected, underscoring the strain's robust resistance mechanisms. The findings underscore the urgent need for targeted surveillance systems to monitor infection sources and manage antibiotic use in healthcare settings to prevent further escalation of AMR.

Key words: Bacteria, *Pseudomonas aeruginosa*, resistance, virulence, semen

1. Introduction

Antimicrobial resistance is thought to be a component of virulence that facilitates the ability of harmful bacteria to cause infections. Shortly after the initial antibiotic categories, penicillin and sulfonamides, were introduced, in the latter part of the 1930s and early 1940s, the initial instances of resistance to antibiotics emerged (Maria et al., 2024). Many bacteria, including *S. aureus* strains, developed record-breaking resistance to various antibiotic families. Resistance to antibiotics is not a novel concept; nonetheless, the quantity of tolerant organisms, the regions where antibiotic resistance is present, and the extent of resistance within one microbe are unparalleled and steadily increasing (Baciu et al., 2024; Chambers and Fowler, 2024; Kurakado et al., 2024).

Antibiotic-resistant strains of previously thought-to-be-controllable illnesses and infectious agents are resurfacing. Nevertheless, viruses, fungi, and parasites are among the numerous microbes that exhibit antibiotic resistance (Mag et al., 2024; Nanyangwe-Moyo et al., 2024). The majority of antibiotics were used in hospitals during the time when bacteria resistant to antibiotics were first detected. In military facilities in the 1930s, *Streptoccus pyogenes* that was tolerant to sulfonamide first appeared. Not long after penicillin was first

introduced in the 1940s, civilian hospitals in London faced the threat of *S. aureus* resistant to the antibiotic. Similar to this, *Mycobacterium tuberclosis* that was resistant to streptomycin surfaced in the public shortly after this antibiotic's discovery (Guerra and LaRock, 2024; Abbasnia et al., 2024). In the late 1950s and early 1960s, initial resistance to multiple medications was observed in intestinal bacteria, specifically *Salmonella*, and *E. coli* (Romero-Rodríguez.et al., 2024). In countries that were developing, these strains caused serious clinical issues and even resulted in fatalities. However, some people, especially those in the developed world, perceived the resistance issue as an interest with minimal health implications, limited to digestive organisms in far-off places (Bennett et al., 2024). This perception was fueled by rising antibacterial use, which caused resistance to rise in a variety of bacteria, particularly in nations that are developing where antibacterial agents were easily accessible without prescriptions. Hygiene issues contributed to the propagation, and limited funding for healthcare hampered the availability of new, costlier but more efficient antibiotics (Boyce, 2024).

The quality of fresh semen is significantly impacted by bacterial infection. Contamination by bacteria can come from either non-animal or animal sources and happens throughout the semen collecting process. Furthermore, most of the bacteria isolated from fresh semen (more than 80%) are Gram-negative (Ďuračka et al., 2023; Thacharodi et al., 2023). Semen can occasionally contain infections like *Pseudomonas aeruginosa* (Ferris et al., 2017) and *Streptococcus equi* (Tyrnenopoulou and Fthenakis, 2023), as well as to the usual flora (Al-Kass et al., 2019). *P. aeruginosa* is a major pathogen responsible for a wide range of hospital-acquired infections, including respiratory tract infections, urinary tract infections, and wound infections. This opportunistic bacterium is known for its ability to develop resistance to a wide variety of antibiotics, making it a significant concern in healthcare settings (Farzin et al., 2023). The high rate of antibiotic resistance in *P. aeruginosa* limits treatment options, leading to prolonged hospital stays, increased medical costs, and a higher risk of complications and mortality. Resistance mechanisms, such as the production of extended-spectrum beta-lactamases (ESBLs), further complicate the clinical management of infections caused by this bacterium (Ur Rahman et al., 2018). Certain resistant bacterial species were identified to carry the blaTEM and blaCTX genes, which are responsible for encoding beta-lactamase enzymes that break down antibiotics like penicillins and cephalosporins, contributing to resistance. Identifying resistance genes, such as blaTEM and blaCTX, is crucial in understanding the molecular basis of antibiotic resistance and developing more effective treatment strategies. Additionally, the bacterial strain was identified using 16S rRNA sequencing, a method that analyzes a specific region of the bacterial RNA to determine its species by comparing it with known sequences in genetic databases. The growing resistance of *P. aeruginosa* underscores the urgent need for comprehensive surveillance and stewardship programs to combat the spread of resistant strains in healthcare environments (Bajaj and Bajaj, 2024).

The novelty of this study lies in the identification of extended-spectrum beta-lactamase (ESBL)-producing *P. aeruginosa* from semen samples in an Egyptian hospital setting, with a focus on resistance genes blaTEM and blaCTX, which play a crucial role in the resistance mechanism. This finding emphasizes the alarming presence of ESBLs in urological samples, which has not been extensively documented in Egypt. The study highlights the urgent need for targeted surveillance systems to monitor and control antibiotic-resistant infections in hospital environments and recommends tailored antibacterial treatments to mitigate resistance risks in healthcare settings. The present investigation focuses on the isolation and identification of pathogenic *P. aeruginosa* in semen. Moreover, the identification of resistance genes contained in the DNA of organisms that are multidrug resistant.

2. Materials and methods

Collection of samples

Patients were referred to or received visits from the Urology department at El-Hussien Hospital. A total of 100 participants were enrolled between

May and June of 2022, consisting of two groups: 50 healthy fertile individuals and 50 individuals with infertility. The participants' age range was 20-45 years, with a history of either normal fertility or infertility, as confirmed by clinical evaluation. The infertile males were used as controls for the seminal fluid analysis bacteriological study, and their semen samples were collected for further testing. The control group, consisting of 50 healthy fertile men, was randomly selected during the study period. Semen cultures were performed on each specimen to detect bacterial contamination and resistance profiles. The following were the inclusion criteria: After a year of consistent, unprotected sexual activity, infertile men from couples who were unable to conceive successfully become parents themselves. Man with azoospermia who is infertile. Man is infertile with both asthenozoospermia and oligozoospermia. The group used as a control consisted of 50 male volunteers who appeared healthy and met similar criteria as the individual in question. The study received ethical approval from the International Islamic Center for Population Studies and Research, Al-Azhar University, no (AZFC2252022).

Isolation and identification of bacteria from seminal fluid

Bacteria were isolated and identified from seminal specimens using the techniques outlined by McVey et al. (2022) and Qin et al. (2022). The expanding colonies were then grown on nutrient agar plates after the specimens had been injected into nutrient broth (McVey et al., 2022). A subculture on solid agar plates using media like MacConkey agar and blood agar (Sigma, Egypt). The pure bacterial strains were obtained through re-cultivation on nutrient agar plates (Sigma, Egypt), where they were incubated for 24 hours at 36°C (Blomquist and Nix, 2021) and then for another 24 hours at 36°C in an aerobic environment (Botelho et al., 2019). Morphological characteristics were demonstrated by Gram staining and KOH of isolated strains (Shouman et al., 2023). Biochemical tests included oxidase, catalase, and urease (McVey et al., 2022).

Antimicrobial and sensitivity testing

All isolates were subjected to sensitivity evaluation for antibiotics using Mueller-Hinton medium, in accordance with the International Standard (Humphries et al., 2018). After letting the medium cool to 45 ˚C, it was poured into petri dishes until the thickness reached around 4 mm. To allow the surplus moisture to escape, the solidified plates were then incubated at 36˚C for 14 to 25 minutes. A sterile swab was dipped into the inoculum to inoculate the plates. Excess inoculum was then removed by pushing and turning the swab firmly towards the tube's side wall over the fluid level. The swab was then wiped over the medium's surface, turning the plate three times at a sixty-degree angle after every use. At last, the swab slipped around the outermost portion of the layer of agar. The plate was covered and allowed air to dry for a few minutes at ambient temperature. To prevent moisture buildup on the agar surface, the antibiotic discs were placed and the agar plates turned upside down for incubation following 15-minutes of inoculation (Andrade et al., 2023). Ten antibiotic discs were chosen, and each was carefully pressed downward to make sure there was adequate contact with the medium before being applied to each dish using heated forceps. Following an overnight incubation period at 37˚C, the length of every zone, including the zone of inhibition, was determined in millimeters and compared to the conventional inhibitory zone (Andrade et al., 2023).

Molecular testing for *P. aeruginosa*

16SRNA identification and phylogenetic analysis

In Luria-Bertani broth (LB), bacterial isolates were incubated at 37°C for 24 hours with constant shaking at 180 rpm. After three rounds of washing by recovery in 0.85% NaCl and centrifugation, the isolates were harvested at 12,000 g for five minutes. Following the directions provided by the manufacturer, genomic DNA was isolated using the Gene JET Genomic DNA cleaning kit (Thermo Scientific, USA). Forward primer 8F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and reverse primer 1492R (5′-GGG CGG GGT GTACAA GGC-3′) were used for amplification under the following conditions: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at

95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. The amplified PCR product was then cleaned using a commercially available PCR cleanup kit (Thermo Scientific, USA) and sequenced. The raw sequencing data were processed and edited using the Finch T.V. 1.4.0 software (Geospiza, USA). The resulting 16S rRNA sequences were analyzed using the National Center for Biotechnology Information (NCBI) BLAST (N) tool (MD, USA) for species identification. Sequence alignment was performed using ClustalW 2.1 (Bioinformatics, UK). Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA X (Kumar et al., 2018) to assess the genetic relatedness of the bacterial isolates.

Molecular detection of resistant genes for *P. aeruginosa*

The blaTEM gene, spreading roughly 866 bp, was amplified using blaTEM-F (5'-ATGAGTATTCAACATTTCCG-3') and blaTEM-R (5'-GACAGTTACCAATGCTTAATCA-3'), as well as the blaCTX gene, which was amplified with blaCTX-F (5'-ATGGTTAAAAAATCACTGCGTC-3') and blaCTX-R (5'-TTGGTGACGATTTTAGCCGC-3'). The PCRs were conducted in 25 µl reaction volumes with 12.5 µl DreamTaq master mix PCR Kit (Thermo Scientific, USA), 0.5µl of each primer, and 3 µl of DNA template, utilizing a thermocycling profile. Denaturation at 95°C for 4 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 minute, were the steps in the reaction PCR protocol. The last extension step was 72°C for 10 minutes. The blaTEM PCR results were amplified, and then they were quantitatively evaluated using 1.5% agarose gel electrophoresis (Peymani et al., 2017).

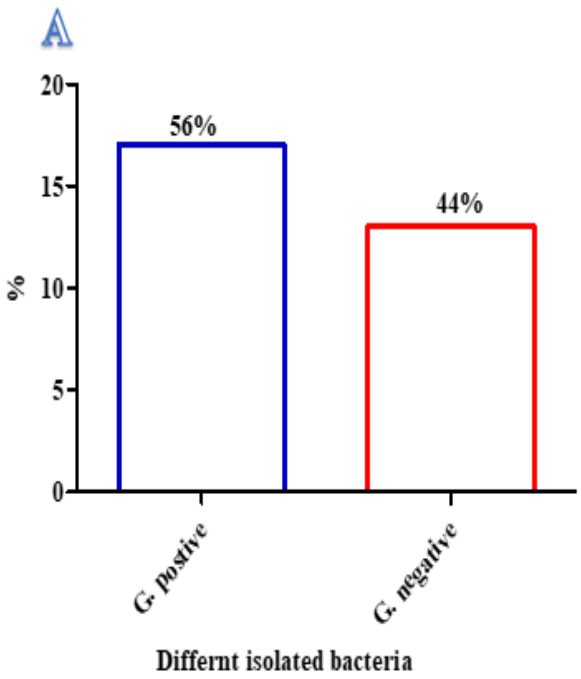
Statistical evaluation

The statistical evaluation of the outcomes was carried out using GraphPad Prism (V5, CA, USA). The results were shown as mean and standard deviation. For every parameter, Pearson's correlation coefficient was utilized to ascertain the association between the variables. When $p< 0.05$, the variations were deemed significant.

Results

Isolation of bacteria from semen

Semen cultures were done on all 100 research participants, 50 sick and 50 healthy controls. Out of the 50 patients, only 30 had a positive semen culture. Of the 30 individuals with bacteriospermia, 17 (56%) had organisms with Gram positive infection, while only 13 (44%) had organisms with gram negative infection, as depicted in Fig. 1b. The most common organism, *P. aeruginosa*, was found in bacteriospermia and accounted for 61.5% of the total (8). The isolate frequencies for Gram positive bacteria were *S. aureaus*, *B. Subtilis*, and *E. faecalis* were 41% (7), 35.3% (6) and 23.7% (4), respectively. While the levels for the gram-negative bacteria were *E. coli*, *P. mirabilis*, and *N. gonorrhoeae* were 23% (3), 7.8% (1), and 7.8% (1), respectively, as depicted in Figure 1b. The distribution of the isolated bacteria demonstrated a highly significant difference ($p< 0.05$) among pyospermia (infection semen) and non-infection semen.



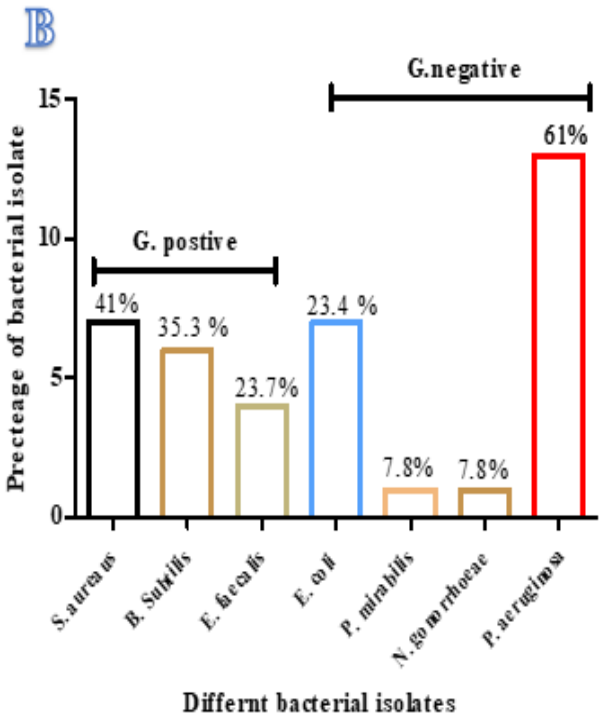


Fig. 1. Different levels of isolated bacteria from semen according to Gram stain (A) and Different identified bacteria and their percentages in both groups of bacteria (B).

Testing antimicrobial and sensitivity

Applying the Kirby-Bauer disc diffusion procedure and the least inhibition level for each bacterial isolate to the most frequently utilized antibacterial drugs for the therapy of pyospermia, the antibiotic sensitivity testing was conducted. All of the gram-positive bacteria were sensitive to most of the applied antibiotics, while some of the gram-negative bacteria were resistant to some of the used antibiotics. Also, it was noticed that *P. aeruginosa* was the most common and resistant bacteria in the collected samples, as demonstrated in Table 1 and Fig. 2.

Fig. 2. Determination of the most resistant bacterial isolate versus all tested antibiotics.

Table 1. Antibiotic and sensitivity for different bacteria isolated from semen

Isolates	E	DA	CEC	NOR	AM	AMC	OX	TE	FOX	TN
G. positive bacteria										
S. aureaus	3±0.1	3±0.3	2.2±0.3	2.4±0.1	2.2±0.1	2.6±0.1	2.1±0.1	2.3±0.1	3±0.2	3±0.3
B. Subtilis	1.8±0.2	1.9±0.2	2.0±0.2	2.1±0.2	2.0±0.2	2.0±0.2	1.9±0.2	1.8±0.2	1.9±0.3	1.7±0.1
E. faecalis	1.9±0.1	2.3±0.1	2.0±0.1	R	3.0±0.1	2.2±0.3	2.0±0.1	2.0±0.2	1.9±0.2	1.8±0.1
G. negative bacteria										
E. coli	1.7±0.3	2.0±0.1	1.9±0.2	R	1.3±0.2	1.3±0.2	1.2±0.1	0.8±0.1	0.7±0.2	1.2±0.2
P. mirabilis	1.6±0.1	1.0±0.2	R	1.6±0.2	1.2±0.2	2.0±0.2	R	1.6±0.1	1.1±0.3	R
P. aeruginoa	R	R	R	R	R	R	R	R	R	R
N. gonorrhoeae	1.1±0.1	R	1.3±0.2	1.4±0.1	1.6±0.1	2.0±0.1	1.3±0.1	1.4±0.2	1.3±0.2	1.4±0.2

Data are tabulated as means ± SD, R: Resistant. E: Erythromycin, DA: clindamycin, CEC: Cefaclor, NOR: Norfloxacin, AM: Amoxicillin, AMC: Amoxicillin-clavulanic acid, OX: Oxacillin, TE: tetracycline, FOX: cefoxitin, TN: Ciprofloxacin. Inhibition zones were represented by mm.

Genetic identification of *P. aeruginosa*

The most drug-resistant bacterial strain could be identified using 16S RNA as *Pseudomonas aeruginosa*, and it has been deposited in the gene bank with the accession number LC455963 (https://www.ncbi.nlm.nih.gov/nuccore/LC455963.1), and it had 100% similarity with the isolates in the gene bank as shown in the phylogenetic tree (Fig. 3).

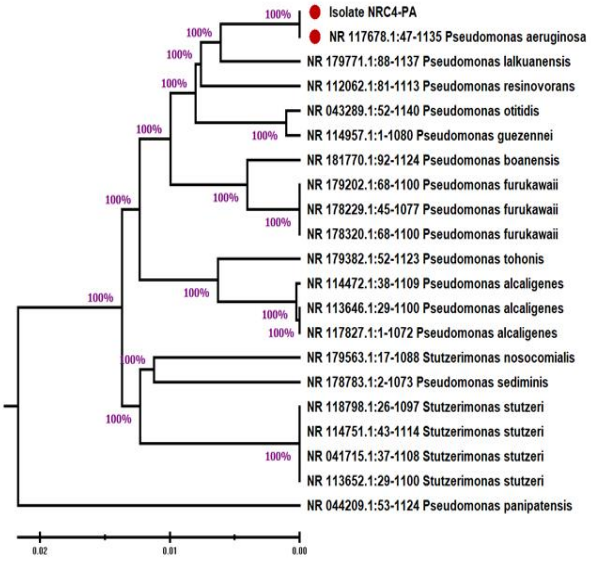


Fig. 3. Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between isolate *P. aeruginosa* strain and other species belonging to the genus *Pseudomonas*. The tree was constructed using the MEGA11 and neighbor-joining method.

Genetic detection of resistant genes from *P. aeruginosa*

Three DNA samples of *P. aeruginosa*. This study tested positive for the blaTEM gene, as shown using PCR gel with the expected amplicon of 866 bp, corresponding to the blaTEM gene (Figure 4). Also, three DNA samples of *P. aeruginosa*. were tested positive for the blaCTX gene with the expected amplicon of 866 bp, corresponding to the blaCTX gene (Fig. 5).

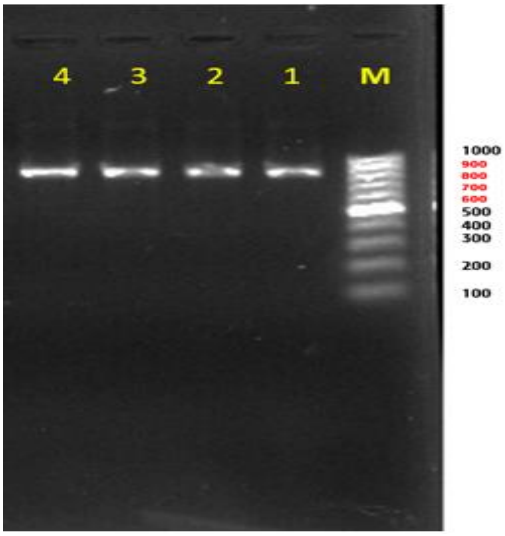


Fig. 4. 1.5% gel electrophoresis of PCR for detection of the blaTEM gene among *P. aeruginosa*. Lane M: Molecular marker (Thermo Scientific™ Gene Ruler 100 bp DNA Ladder); Lane 1 to 4: *P. aeruginosa* isolates. The expected 866-base pair fragment was amplified corresponding to the blaTEM gene.

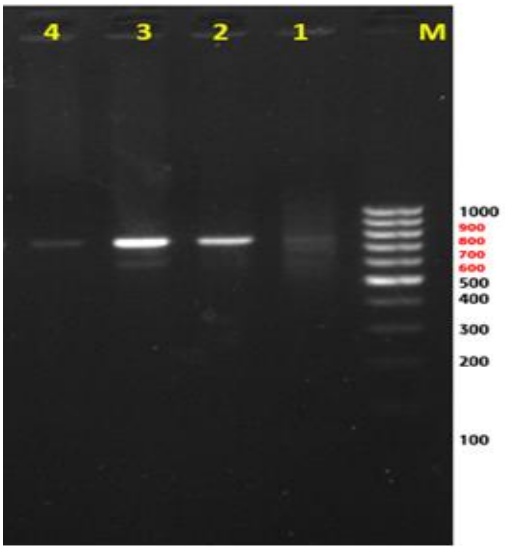


Fig. 5. 1.5% gel electrophoresis of PCR for detecting the blaCTX gene among *P.aeruginosa*. Lane M: Molecular marker (Thermo Scientific™ Gene Ruler 100 bp DNA Ladder); Lane 1 to 4: *P. aeruginoa* isolates. The expected 866-base pair fragment was amplified corresponding to the blaCTX gene.

4. Discussion

The results of the investigation demonstrated that thirty of the subject group's samples had positive bacterial cultures, and 20 of the subject group's specimens had no growth of bacteria following 48 hours of development. This could be because other causal agents, such as Chlamydia or Mycoplasma, were present and may require a particular method for identification. These findings are aligned with those published by Abbas et al. (2023), who demonstrated a connection among patients' aberrant semen pattern and bacteriospermia. A number of diverse characteristics of sperm showed a considerable beneficial response to antibiotic therapy, as validated by Anel-Lopez et al. (2021). Additionally, Henkel (2024) observed similar findings in different populations, indicating that bacteriospermia is a significant contributor to infertility across diverse regions. The current findings were consistent with previous research that demonstrated the relationship between infertility and the bacteriospermia incidence frequency (Corral-Vazquez et al., 2024; Grande et al., 2024; Neto et al., 2024). It was reported that seminal fluid samples included Gram-positive organisms. It was discovered that the pathophysiology of persistent pelvic pain syndrome involved Gram-positive organisms (Cohen et al., 2022). They were discovered in the urethral duct walls' focus colonies (Childers et al., 2021). According to Bergman's research, 43% of those diagnosed with prostatitis signs had a substantial number of Gram-positive species (Bergman, 2011). For instance, Kline and Lewis (2016) reported similar observations of Gram-positive organisms in seminal fluid, reinforcing the importance of considering these pathogens in male infertility.

The current report illustrates the existence of various strains of gram-negative bacteria *E. coli*, *P. mirabilis*, and *N. gonorrhoeae*, as well as *P. aeruginosa*. In accordance with Solomon and Henkel (2017) who explained the relation among infertility and Gram-negative bacteria identified in semen. Furthermore, Marchiani et al. (2021) demonstrated the predominance of *P. aeruginosa* and *E. cloacae* in semen specimens. For example, Pacheco et al. (2023) found similar Gram-negative bacterial profiles in male infertility patients, highlighting the global significance of P. aeruginosa in urological infections.

P. aeruginosa, is a significant aggressive pathogenic bacterium that is linked to several illnesses related to healthcare (Scheeren et al., 2020; Veetilvalappil et al., 2022). Numerous antibiotic agents are clinically resistant to *P. aeruginosa* (Rivera et al., 2022). Worldwide recognition of nosocomial diseases caused by MDRPA strains is growing, and these infections are linked to higher rates of morbidity, death, and therapy costs (El Aila et al., 2024; Abou Elez et al., 2024). Among Gram-negative bacteria, ESBLs are one of the key factors that contribute to ß-lactam antibiotic susceptibility (Carvalho et al., 2022). The predominant genetic groupings of ESBLs in clinically relevant Gram-negative bacteria are CTX-M (Bartlett et al., 2024; Khachab et al., 2024). Studies by Sharma et al. (2024) and Arulkumaran et al. (2020) have highlighted the rising prevalence of ESBL-producing bacteria, emphasizing the urgent need for more effective antibiotic stewardship programs worldwide.

The present results revealed the presence of blaTEM as well as blaCTX in the resistant bacteria from semen. A significant worry concerning the growing number of *P. aeruginosa* that produces ESBLs in hospital environments is the inability to cure infections brought on by this organism because of restricted treatment options (Bakthavatchalam et al., 2024; Bandić Pavlović et al., 2024). The existence of blaSHV-12 was demonstrated by Uemura et al. in Japan in *P. aeruginosa* isolates taken from burned individuals (Uemura et al., 2010). Besides, according to Polotto et al. (2012) the most common ESBL gene in Brazil was blaCTX-M-2 (19.6%). All of these facts point to the ESBL-encoding genes' effective global expansion. Aslan and Akova (2019) and Bush and Bradford (2020) reported similar findings, reinforcing the need for integrated approaches to combat the spread of ESBL-producing pathogens across healthcare settings.

Conclusion

This study highlights the issue of *Pseudomonas aeruginosa* resistance to antibiotics used to treat seminal fluid infections in Egyptian hospitals, where the isolates showed high resistance to common antibiotics. It was found that these isolates contain the blaTEM and blaCTX genes, which produce ESBL enzymes that break down beta-lactam antibiotics, making it difficult to treat infections with conventional therapies. The presence of these genes in clinical isolates presents challenges on both therapeutic and epidemiological levels. In addition to limiting available treatment options, these genes contribute to the spread of resistant infections within hospitals, increasing healthcare costs and patient suffering. Epidemiologically, the spread of these genes heightens the risk of antibiotic-resistant infections, threatening public health. The results underscore the importance of implementing strict monitoring systems in hospitals to track the spread of resistant strains and develop effective strategies to control infections. The study also recommends raising awareness among medical staff and the public about the optimal use of antibiotics and avoiding excessive and unnecessary use, which will help reduce the emergence of resistant strains. Addressing this issue requires integrated collaboration between healthcare providers and policymakers to maintain the efficacy of antibiotics and ensure continued successful treatment in the future.

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