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Original Article

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Recovered from Disinfectants Often Used in Khartoum Tertiary Care Hospitals, *Pseudomonas aeruginosa* Isolates are able to Produce β-lactamase Resistance Enzymes

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Abstract

Background and study aim: Disinfectants play a crucial role in infection control in hospitals, but some bacteria can resist these disinfectants or contaminate them. This study aims to investigate the potential disinfectant contamination in Sudanese hospitals by *Pseudomonas aeruginosa (P. aeruginosa)*. ***Methods:*** The study period for this cross-sectional investigation was between November 2020 and October 2021. The actual collection of disinfectant samples was over a three-week period within that timeframe. Analysis was conducted on 44 disinfectant samples from nine hospitals in Khartoum state. The 44 disinfectant samples collected encompassed 9 distinct types of disinfectants commonly used in hospital settings. The presence of *P. aeruginosa* was determined using a combination of microbiological methods and genotypic techniques based on Polymerase Chain Reaction (PCR). The antibiotic susceptibility of bacterial isolates was assessed using the disc diffusion method. Additionally, PCR was used to identify a number of ß-lactamase genes. ***Results:*** Among 44 disinfectant samples, 7 samples (15.9%) tested positive for *P. aeruginosa* contamination. Contamination was most frequently observed in chloroxylenol-based disinfectants (5/9), followed by sodium hypochlorite (1/6) and chlorhexidine gluconate–cetrimide formulations (1/1). *P. aeruginosa* showed resistance to both the cephalosporin and penicillin groups of antibiotics, and all isolates tested positive for the blaTEM-1 resistance gene. ***Conclusions:*** Significant contamination with *P. aeruginosa* was found in the disinfectant samples examined, posing a risk to hospital patients.

Keywords: ***Disinfectants; Nosocomial infection; Pseudomonas aeruginosa; Resistant bacteria; β-lactamase.***

Introduction

A disinfectant is a chemical agent that kills microorganisms on nonliving objects [1]. They play a crucial role in infection control procedures and aid in the prevention of nosocomial infection [2]. One of the causes of hospital infection that has been documented is the use of contaminated disinfectants. Research has shown that both commercially sold stock solutions and prepared diluted disinfectants used in hospitals have been exposed to contamination [3]. One study reported that disinfectant solutions, particularly aqueous chlorhexidine used hospital-wide, were contaminated with live Gram-negative bacteria, specifically *Pseudomonas* species [4]. Gram-negative bacilli exhibit greater resistance to disinfectants than Gram-positive bacteria [5].

Pseudomonas aeruginosa (P. aeruginosa) is a gram-negative, rod-shaped, aerobic bacterium from the family Pseudomonadaceae. It is a major global health concern due to its role in hospital infections, high morbidity and mortality, and multidrug resistance [6].

P. aeruginosa demonstrates resistance to disinfectants [7,8] and high salt concentrations, and it is also able to endure temperatures between 4 and 42°C [9]. Additionally, it produces distinctive water-soluble pigments, such as pyocyanin (blue-green), pyoverdine (yellow and fluorescent), pyomelanin (brown), and pyorubin (red), which aid in identification, though some strains do not produce pigment [10,11].

At the molecular level, Polymerase Chain Reaction (PCR) has been used extensively as a fast and accurate technique for identifying *P. aeruginosa* because of its high specificity and sensitivity [12]. The outer membrane protein (oprL) contributes to *P. aeruginosa*'s innate resistance to antibiotics and disinfectants. As this protein is unique to this organism, it has been widely used as a reliable tool for the quick identification of *P. aeruginosa* [13,14]. As with other Gram-negative rods, *P. aeruginosa* is well known for

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producing β-lactamase enzymes, which serve as the main mechanism of resistance [15,16]. At least 120 of the more than 800 β-lactamases found in gram-negative bacilli have been identified in *P. aeruginosa*. These enzymes hydrolyze the β-lactam ring in β-lactam antibiotics to produce inactive compounds [17,18]. Based on how similar their amino acid sequences are (Ambler classification), these enzymes can be categorized into four groups (A, B, C, and D) [19,20]. Class A include Temoneira (TEM), cefotaximase (CTX-M), sulfhydryl variable (SHV), and Vietnam extended-spectrum β-lactamase (VEB), while OXA is a class D enzyme (oxacillin hydrolyzing enzyme) [21]. TEM-1 primarily hydrolyzes ampicillin but has little effect on extended-spectrum cephalosporins. BlaVEB provides high resistance to (ceftazidime, cefotaxime) and aztreonam. SHV enzymes show strong resistance to ceftazidime, while remaining sensitive to cefotaxime [21,22]. CTX-M enzymes are named because of their hydrolytic activity against cefotaxime [18]. On the other hand, *P. aeruginosa* possesses oxacillin-hydrolyzing (OXA-type) β-lactamases. They initially had a preference for penicillins, but some of them evolved to resist extended-spectrum cephalosporins (cefotaxime, ceftazidime) and aztreonam [23].

Pseudomonas aeruginosa is a common cause of hospital-acquired infections and is known for its resistance to disinfectants. β-lactamase-harboring *P. aeruginosa* exhibits a strong drug resistance pattern against different groups of antibiotics and significantly endangers public health. The objectives of this study are to examine various disinfectant samples obtained from different hospitals in Khartoum for their contamination with *P. aeruginosa*, to study the antimicrobial resistance profile of *P. aeruginosa* isolates, and to detect the existence of various β-lactamase resistance genes (blaTEM-1, blaSHV, blaCTX-M, blaVEB, blaOXA-1).

Materials and Methods

Sample collection

A cross-sectional laboratory-based study was carried out between November 2020 and October 2021. Disinfectant samples were collected over a period of three weeks within that timeframe. A total of 44 disinfectant samples (5 ml each) of working dilutions of disinfectants commonly used at nine Khartoum tertiary hospitals in Sudan were collected in small sterile bottles using sterile syringes and transferred to the microbiology laboratory at the Central Research Laboratory, Ministry of Higher Education and Scientific Research, Khartoum, Sudan. Hospitals were selected using convenience sampling, representing the major hospitals in Khartoum. The following information was obtained for each disinfectant sample: the product name, ingredients, time and method of preparation, and expiration dates.

Isolation and microbiological identification of *P. aeruginosa*

Each sample was cultured on cetrimide agar plates (HiMedia, India) (triplicate), then incubated aerobically at 37°C for 24 h (Sanyo, Japan). The isolates were identified through conventional bacterial identification methods, including Gram stain reaction, pigment production on selective cetrimide agar medium, and positive oxidase, catalase, and motility tests [24].

Antibiotic susceptibility testing

All bacterial isolates were tested for antibiotic susceptibility in triplicate using the disk diffusion technique [25] on Mueller-Hinton agar medium (HiMedia, India). A total of 24-h-old tested isolates were picked up with a sterile wire loop and suspended in sterile normal saline. A sterile cotton swab was used to inoculate the suspension onto a plate of Mueller-Hinton agar after the suspension's turbidity had been adjusted to 0.5 McFarland's standard. After evenly swabbing the medium's surface with the swab, antibiotic discs (HiMedia, India) were added. The plate was incubated aerobically for 18 h at 37°C. According to Clinical Laboratory Standards Institute (CLSI) guidelines, the diameters of the inhibition zones were measured in millimeter (mm) and interpreted. A total of six antimicrobial agents (HiMedia, India), representing three different classes of the ß-lactam antibiotics (penicillins, cephalosporins and monobactams) were used at the following concentrations: ampicillin (AM) 10 µg, ceftriaxone (CRO) 30 µg, cephalexin (CL) 30 µg, cefotaxime (CTX) 30 µg, ceftazidime (CAZ) 30 µg, and aztreonam (ATM) 30 µg.

Molecular analysis of bacterial isolates

DNA extraction

DNA was extracted using the boiling method [26] with some modifications as follows: three to five fresh colonies of bacterial isolates were added to 1.5 ml of deionized water in an Eppendorf tube, which was pelleted using a centrifuge at 10000 rpm for 2 min, and the supernatant was removed. The cell pellet was suspended in 200 µl of deionized water, then the tube was heated in a water bath at 95°C for 10 min. The tube was mixed with a vortex mixer for 10 s, then it was cooled to room temperature and centrifuged for 2 min at 10000 rpm. Following centrifugation, the supernatant was transferred to a sterile 1.5 mL Eppendorf tube. Extracted DNA was quantified using a NanoDrop Lite spectrophotometer (Thermo Scientific, USA), and the quality was estimated from the ratio of absorbance at 260/280 nm. Extracted DNA was stored at −20°C.

PCR amplification and gel electrophoresis

Species-specific PCR targeting the outer membrane lipoprotein gene oprL was used for molecular identification of *P. aeruginosa* (**Table 1**). The PCR amplification was conducted using 90 ng of template DNA, 0.5 µl of each primer (10 µM) in a Maxime PCR premix kit (iNtRON Biotechnology, Korea), and up to a final volume of 20.0 µl with deionized water. *P. aeruginosa* ATCC 27853 was used as a positive control, and deionized water as a negative control. Then, five primer pairs (**Table 1**) were used for the detection of β-lactamase resistance genes (blaTEM-1, blaSHV, blaCTX-M (Eurofins Genomics, Germany), and blaVEB and blaOXA-1 (Macrogen, Korea)). A PCR reaction was conducted using 250 ng of template DNA, 0.5 µl of each primer (10 µM) in a Maxime PCR premix kit (iNtRON Biotechnology, Korea), and deionized water to a final volume of 20.0 µl. The reaction mixture was amplified using a thermal cycler (Applied Biosystems, USA).

The amplified products were analyzed on a 1.5% agarose gel (iNtRON, Korea). 1X Tris-borate-EDTA buffer (TBE) solution (iNtRON Biotechnology, Korea) was used to prepare the agarose gel. The mixture was heated until boiling, then 2 µL ethidium bromide (0.5 µg/ml) was added, and the solution was left to cool. After comb fixation, the agarose gel was poured into the casting tray and allowed to solidify at room temperature. After carefully extracting the comb, the gel casting tray was positioned within the electrophoresis tanks containing 1X TBE buffer. 5 µL of PCR products were loaded into each comb well, and 5 µl of 100-1500 bp DNA ladder (Ampigene, India) was added into one well. To separate products, electrophoresis was carried out at 100V and 60A for 50 min using the Enduro electrophoresis system (Labnet, USA). Specific DNA bands from the gel were visualized using the gel documentation system (ENDURO, USA) [27].

Table 1: Primers used in this study and amplification conditions.

Target gene, product size, and reference	Primer sequence (5′ to 3′)	PCR conditions	No. of cycles
OprL (504 bp) [14]	F:ATGGAAATGCTGAAATTCGGC R:CTTCTTCAGCTCGACGCGACG	94°C/5 min 94°C/1 min 57°C/1 min 72°C/1 min 72°C/10 min	30
TEM-1 (445 bp) [21]	F:TCGCCGCATACACTATTCTCAGAATGA R:ACGCTCACCGGCTCCAGATTTAT	94°C/10 min 94°C/ 40 s 63°C for 40 s 72°C/1 min 72°C/7 min.	30
SHV (747 bp) [21]	F:TGCTTTGTTATTCGGGCCAA R:ATGCGTTATATTCGCCTGTG	95°C/1 min 94°C for 30 s 56°C/30 s 72°C/80 s 72°C/5 min	35
CTX-M (593 bp) [21]	F:ATGTGCAGCACCAGTAAAGTGATGGC R:TGGGTAAAGTAAGTGACCAGAATCAGCGG	95°C/3 min 95°C/30 s 65°C/30 s 72°C/1 min 72°C/5 min	30
VEB (647 bp) [28]	F:CATTCCCGATGCAAAGCGT R:CGAAGTTTCTTTGGACTCTG	95°C/1 min 94°C/30 s 56°C/30 s 72°C/80 s 72°C/5 min.	35
OXA-1 (564 bp) [28]	F:GGCACCAGATTCAACTTTCAAG R:GACCCCAAGTTTCCTGTAAGTG	94°C/5 min 94°C/30 s 59°C/30 s 72°C/1 min 72°C/7 min	35

bp: Base pair, OprL: Outer membrane protein, blaTEM-1: temoniera β-lactamase-1, blaCTX-M: cefotaximase, blaSHV: sulphydril variable β-lactamase, blaVEB: Vietnamese extended spectrum β-lactamase, OXA: Oxacillin hydrolyzing enzyme.

Statistical analysis

The antimicrobial susceptibility test results were analyzed and plotted using Microsoft Excel 2010. We compared the percentage prevalence of *P. aeruginosa* isolates and their sensitivity, resistance, and intermediate resistance to different antimicrobial agents.

Results

Types of disinfectants frequently used and detection of *P. aeruginosa*.

The types of disinfectants sampled in this study were alcohol, sodium hypochlorite (Sod. hypochlorite), chlorine tablets, chloroxylenol, chlorhexidine gluconate and cetrimonium bromide (CHG and CB), formaldehyde, povidone iodine, hydrogen peroxide (Hyd. Peroxide), and quaternary ammonium chloride (Qua amm ch cpds) (**Figure 1**). They were collected from the general ward, surgical ward, neonatal ward, and asthma room.

Out of the 44 disinfectant samples that were collected and cultured on selective cetrimide agar media, seven samples successfully showed bacterial growth, representing 15.9% of the total samples and coded as (S1, S7, F3, F7, O5, A3, B2), whereas no bacterial growth was detected in the remaining samples. Of the seven contaminated samples, five were chloroxylenol, one sample each of sodium hypochlorite, and chlorhexidine gluconate and cetrimonium bromide.

On selective cetrimide media, five isolates produced yellow-green pigment (pyoverdine), one isolate produced blue-green pigment (pyocyanin), and one isolate showed no pigment production. Considering both conventional methods, such as Gram staining, culture, and biochemical tests (**Figure 2**), and molecular methods (**Figure 3**), all isolates were identified as P. eruginosa.

Antimicrobial susceptibility tests showed various degrees of resistance.

The results of antimicrobial susceptibility testing revealed complete resistance to ampicillin and cephalexin (100%). Additionally, cefotaxime exhibited (85%) resistance rate, while ceftriaxone showed a (57%) resistance rate. On the other hand, there was high susceptibility to ceftazidime (100%) and a fair one to aztreonam (71.5%) (**Figure 4**).

All isolates were found to carry the TEM-1 resistance gene.

Regarding the molecular detection of β-lactamase genes, all isolates were found to carry the TEM-1 gene (**Figure 5**). However, none of the isolates tested positive for CTX-M, SHV, VEB, or OXA-1.

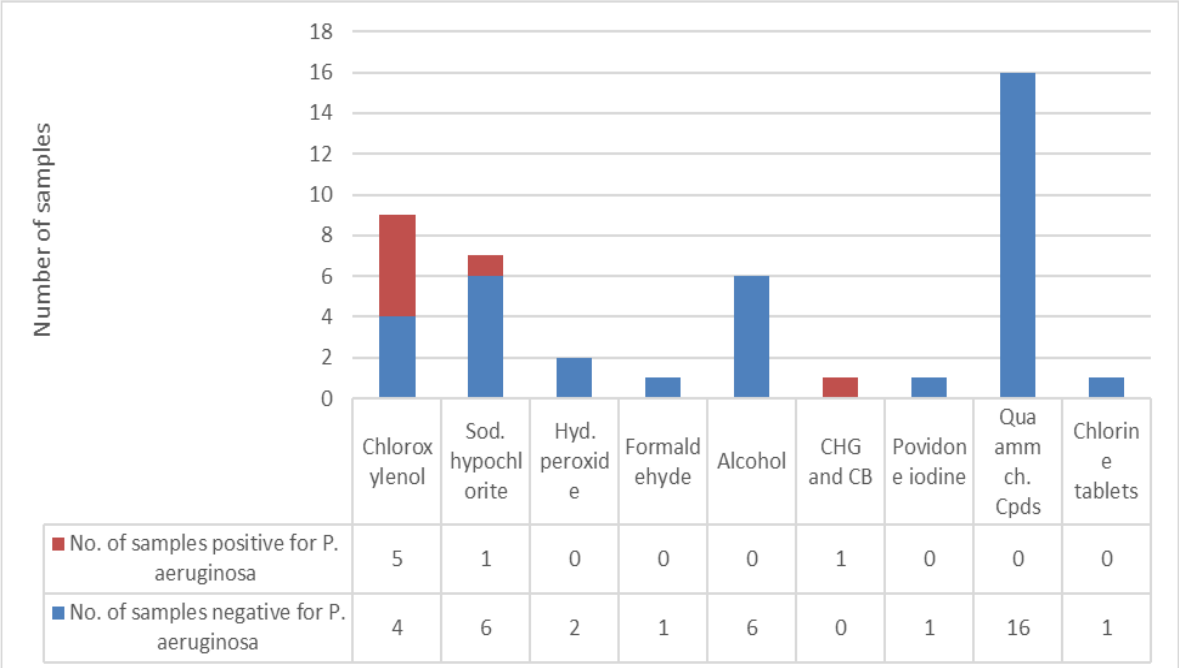

Figure 1: Contamination rate of freshly prepared working diluted disinfectants from different hospitals.

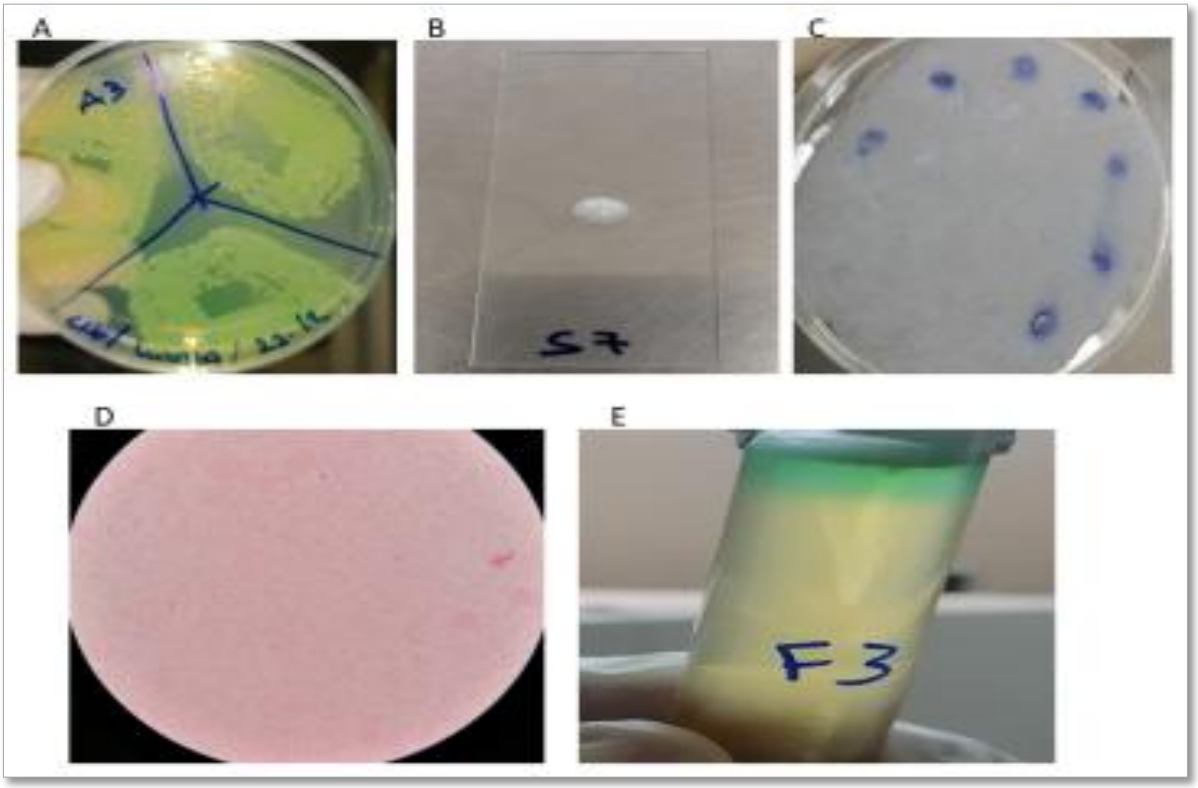

Figure 2: Phenotypic identification of P. aeruginosa. (A) Pigment production, (B) catalase test, (C) oxidase test, (D) Gram staining, (E) motility test.

Table 2: Quantification and qualification of DNA extracted from *P. aeruginosa* isolates.

Samples	DNA concentration ng/µl	DNA purity(A260/A280)
S1	488	1.82
S7	279	1.86
F3	104	1.89
F7	227	1.97
O5	216	1.86
A3	241	1.85
B2	227	1.99
PC	159	1.99

PC: Standard *P. aeruginosa*.

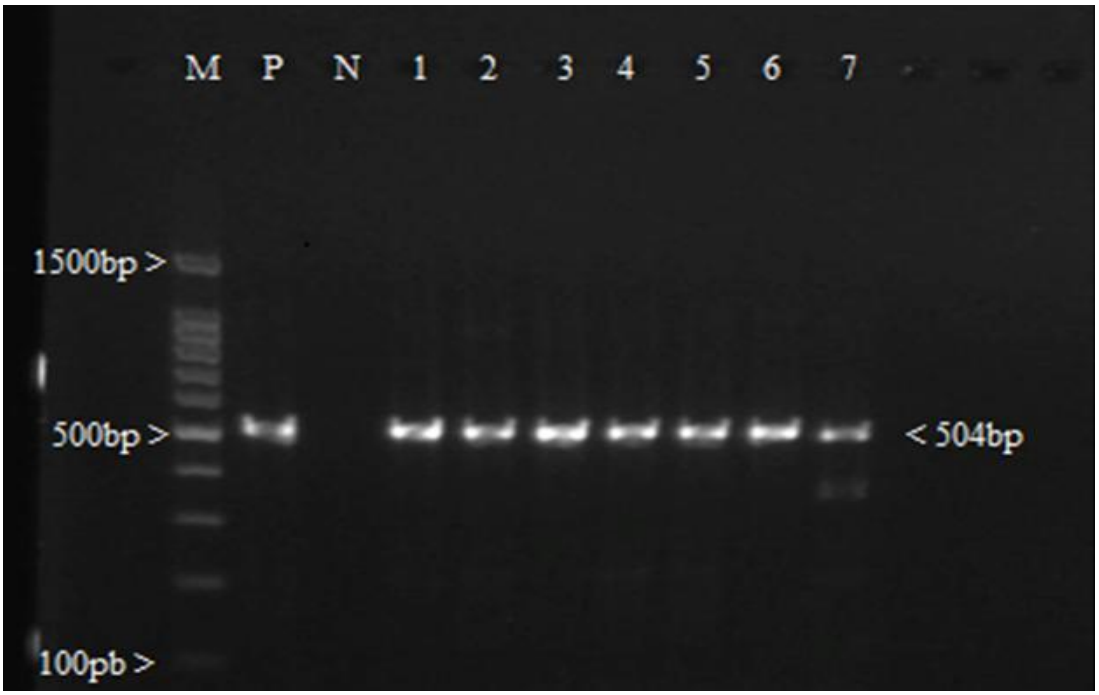

Figure 3 : The oprL gene PCR products (504 bp) were visualized using gel electrophoresis. M= 100-bp DNA marker, P= *P. aeruginosa* positive control, N= Deionized water negative control, lanes 1–7 = *P. aeruginosa* isolates

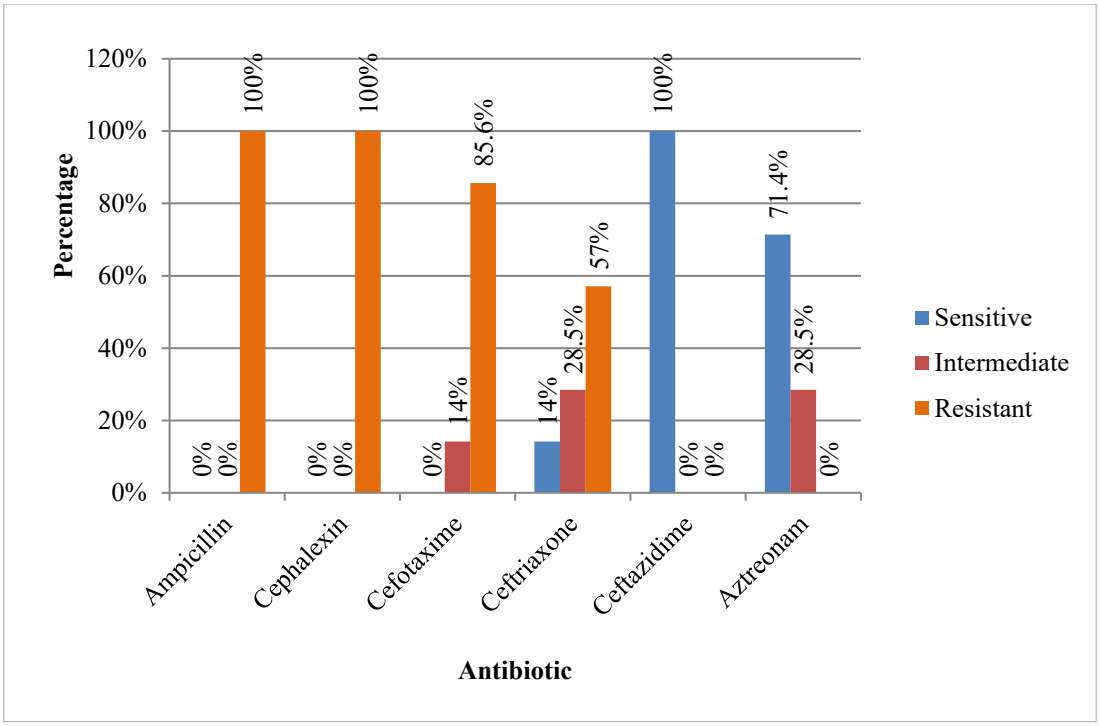

Figure 4: Susceptibility of *P. aeruginosa* isolates to different anti-bacterial agents.

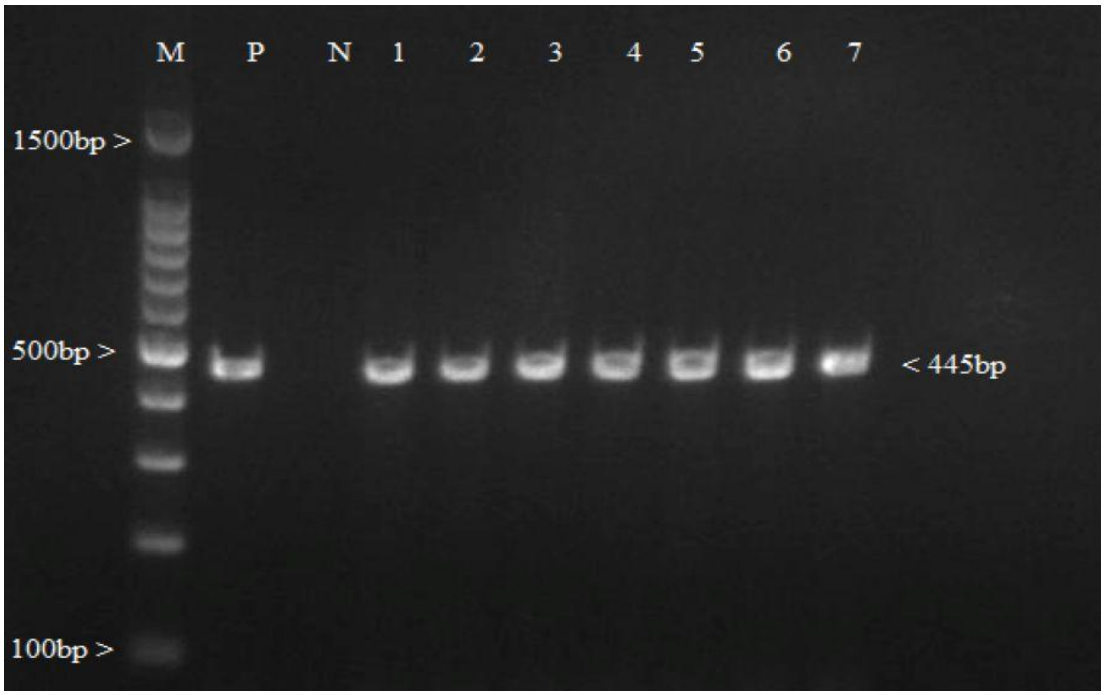

Figure 5: The blaTEM-1 gene PCR products (445 bp) were visualized using gel electrophoresis. M= 100-bp DNA marker, P= *P. aeruginosa* positive control, N= Deionized water negative control, lanes 1–7 = *P. aeruginosa* isolates.

Discussion

Microbial contamination of disinfectants in hospitals is critical because it poses a significant risk to patients' health. Several studies have revealed microbial contamination of disinfectants, with gram-negative non-fermentative organisms [29], specifically *Pseudomonas* sp, being the most commonly isolated [30,31]. This study reports a high rate of contamination (15.9%) of disinfectants obtained from nine different Khartoum hospitals compared with the 6.1% prevalence of contaminated disinfectant samples from the four Trinidad hospitals [31] and the 3% reported in 11 Danish hospitals [32]. A high rate of contamination was observed in chloroxylenol. It represents 71.4% (5/7) of contaminated samples. The presence of *P. aeruginosa* in disinfectant working solutions highlights gaps in hospital infection control guidelines, which should explicitly address disinfectant preparation, dilution, and storage practices. Although expiry dates and recommended guidelines were followed, contamination likely arose from handling practices such as repeated tapping up of containers or use of non sterile water [33,34]. This underscores the need for staff retraining to ensure accurate dilution, proper container management, and adherence to standardized procedures. Moreover, the risk posed by contaminated disinfectants is particularly severe for immunocompromised patients, including those in neonatal and surgical wards. Exposure to resistant *P. aeruginosa* in these settings can lead to serious hospital-acquired infections with limited therapeutic options. Strengthening disinfectant control, staff education, and targeted surveillance in high-risk units are therefore essential to mitigate these risks.

According to the results of phenotypic and biochemical tests, all isolates were confirmed to be *P. aeruginosa*. For more accuracy, the PCR method was employed to specifically target the oprL gene for molecular detection of *P. aeruginosa*, as this protein is found only in this organism. The oprL gene has been reported to be conserved in *P. aeruginosa* [35]. Several studies used oprL as a molecular marker for molecular identification of *P. aeruginosa* in the environment and hospitals [14,36-38]. Now, no studies have been conducted on the detection of *P. aeruginosa* in disinfectants using the molecular marker oprL gene. Other methods were used, but as far as our understanding goes, this represents the first report on the detection of *P. aeruginosa* in disinfectants using conventional and molecular methods.

The results of DNA quantification and qualification (**Table 2**) showed that the boiling method was a highly effective method for DNA isolation, since good yield and purity were obtained.

According to PCR results of the oprL gene, the oprL gene amplicon was detected in all the phenotypically identified isolates, including the non-pigment-producing isolate. As a result, the molecular method and phenotypic and biochemical detection techniques completely agreed.

The fact that *P. aeruginosa* strains recovered here are resistant to multiple antibiotics is concerning. All the isolates (100%) were ampicillin- and cephalexin-resistant. Furthermore, 85.7% and 57.1% of the isolates were cefotaxime- and ceftriaxone-resistant, respectively. All of these isolates are, on average, classified as multidrug resistant if they develop resistance to another class of antibiotics [39]. Actually, it is possible they are already resistant to other classes, but we did not test them. The high prevalence of this resistance against β-lactam agents could be explained by the wide use of these drugs for empirical therapy and misuse of antibiotics in Sudan [40].

Resistance of isolates to ampicillin, cephalexin, and third generation cephalosporins (cefotaxime and ceftriaxone) typically suggests β lactamase production. We found the TEM-1 gene in all isolates (100%). TEM-1 is one of the most common plasmid-mediated β-lactamases and is known to confer resistance to penicillins and early-generation cephalosporins (cephalexin). The presence of this gene in all isolates indicates a strong selective pressure favoring strains harboring this gene, most likely due to the widespread use of β-lactam antibiotics such as ampicillin and cephalexin in clinical settings. No evidence was found to support the presence of any of the other resistance genes (CTX-M, VEB, SHV, and OXA-1). This discrepancy suggests that alternative mechanisms may be responsible for the observed resistance. The possible explanations include overexpression of chromosomal AmpC β lactamases, which are common in *P. aeruginosa* and

capable of hydrolyzing cephalosporins, the presence of other β lactamases families not included in this study, or non-enzymatic mechanisms such as efflux pump overactivity and porin loss, which reduce antibiotic uptake [20]. Accordingly, we recommend future molecular testing to provide a more comprehensive understanding of these resistance patterns.

In conclusion, our study found that 15.9% of the tested disinfectants were contaminated with *P. aeruginosa*, which is considered unacceptable and poses a risk to hospitalized patients. Antimicrobial sensitivity tests exhibited a significant occurrence of resistance among these isolates. Also, our study found that β-lactamase (TEM-1 gene) was found in all samples tested. The spread of these β-lactamase-producing bacteria could lead to complications in antimicrobial therapies. To effectively track the dissemination of this pathogen, employing diverse strategies is crucial.

Declaration

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Conflict of interest

The authors declare no conflict of interest.

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None

Author Contributions

Rehab conceptualized the idea. Lubaba collected the samples and conducted the experiments. Lubaba and Rehab analyzed and interpreted the data. Rehab supervised the research. Lubaba wrote and prepared the original draft. Rehab reviewed and edited the manuscript. All authors read and approved the final manuscript.

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