

Monitoring and Investigation of Resistance Genes *gyrA*, *parC*, *blaZ*, *ermA*, *ermB*, and *ermC* in *Staphylococcus saprophyticus* Isolated from Urinary Tract Infections in Mazandaran Province, Iran

ARTICLE INFO

Article Type Original Article

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How to cite this article

Amiri R, Alipour A, Khajir Engasi A, Amiri AR, Mofarrah R. Monitoring and Investigation of Resistance Genes *gyrA*, *parC*, *blaZ*, *ermA*, *ermB*, and *ermC* in *Staphylococcus saprophyticus* Isolated from Urinary Tract Infections in Mazandaran Province, Iran. Infection Epidemiology and Microbiology. 2023;9(2): 117-125.

Article History

Received: February 21, 2023
Accepted: July 30, 2023
Published: August 19, 2023

ABSTRACT

Background: Bacterial urinary tract infections are observed in all age groups due to the development of antibiotic-resistant species. This study aimed to investigate resistance genes gyrase subunit A (*gyrA*), topoisomerase IV (*parC*) subunit gene, beta lactamase (*blaZ*), erythromycin ribosome methylase (*ermC*), *ermB*, and *ermA* in *Staphylococcus saprophyticus* isolated from patients with urinary tract infections (UTIs) in Mazandaran Province, Iran.

Materials & Methods: In this cross-sectional descriptive study, 3280 clinical samples were collected from patients with UTIs in Mazandaran Province from April to December 2022. Isolates were identified by biochemical tests. Microbial sensitivity tests were performed by disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Polymerase chain reaction (PCR) was used to check the presence of resistance genes.

Findings: Out of a total of 3280 clinical samples collected, 2088 samples were detected by biochemical tests at the genus level. *Escherichia coli* (55.22%) and staphylococci (21.59%) were the most frequent bacterial isolates. *S. saprophyticus* was identified in 52 (2.49%) samples. The frequency of *gyrA* and *parC* genes in *S. saprophyticus* isolates was 23 and 1.92%, respectively. The *blaZ* gene was observed in none of the samples. The prevalence of *ermA*, *ermB*, and *ermC* genes was 21, 1.92, and 26%, respectively. The antibiogram test showed that the highest frequency of resistance to erythromycin, azithromycin, and clarithromycin was 70, 36, and 20%, respectively.

Conclusion: According to the present study findings, rapid detection of these strains in hospitals leads to more effective control of the spread of these strains.

Keywords: Urinary tract infection, *Staphylococcus saprophyticus*, Antibiotic resistance, Resistance genes.

CITATION LINKS

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Introduction

Staphylococcus saprophyticus is an important cause of urinary tract infection (UTI) in young women. This bacterium is one of the coagulase-negative staphylococci resistant to novobiocin, which has recently been emphasized for the first time on its importance in urinary infections [1]. *S. saprophyticus* is thought to be the cause of 10 to 20% of UTIs, and sexually active women are more likely to develop the illness. Cystitis is the most common type of UTI [2]. *S. saprophyticus* has been isolated from meat, cheese, vegetables, animal and human intestines, as well as the environment [3-4]. Quinolones are one of the synthetic antibiotics that are widely used worldwide. Urinary tract infections (UTIs) were previously treated with first-generation (acidic) quinolones, including nalidixic acid. But over time, the range of their effectiveness has improved with changes in subsequent generations. One of these changes was the addition of a fluorine atom at the C-6 position of antibiotic molecules, leading to a broad-spectrum potent activity against different Gram-negative bacteria [5-7]. Fluoroquinolones bind to topoisomerase II (DNA gyrase) and topoisomerase IV (*parC* and *parE*) and impede their activity. DNA gyrase is composed of two subunits A and two subunits B, which are encoded by *gyrA* and *gyrB* genes, respectively [1]. Numerous strategies are used by bacteria to defend themselves against quinolones' antibacterial properties. Plasmid-mediated quinolone resistance (PMQR) mechanisms and chromosomal mechanisms mediated by mutations in genes producing quinolone targets (DNA gyrase and topoisomerase IV) are examples of such mechanisms [8]. Quinolone resistance is associated with mutations in the target enzymes (DNA gyrase and topoisomerase IV), each consisting of two subunits (*GyrA* and *GyrB* in DNA gyrase and *ParC* and *ParE* in topoisomerase IV). The majority of the muta-

tions are found in quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* genes. Three clusters of genes are linked to PMQR, comprising quinolone resistance (*qnr*) genes; *aac(6')-Ib-cr*; and *oqxAB*, *qepA1*, and *qepA2* [9]. The pentapeptide repeat protein family, which includes *qnr* genes (*qnrA*, *qnrB*, *qnrS*, *qnrC*, and *qnrD*), protects DNA gyrase and topoisomerase IV from quinolone inhibition. The *aac(60)-Ib-cr* gene encodes an aminoglycoside acetyltransferase that causes less sensitivity to norfloxacin and ciprofloxacin by antibiotic acetylation. The *oqxAB*, *qepA1*, and *qepA2* genes encode efflux pumps that reduce the intracellular concentration of quinolones [2].

Objectives: The purpose of this study was to investigate the resistance genes *gyrA*, *parC*, *blaZ*, *ermC*, *ermB*, and *ermA* in *S. saprophyticus* strains isolated from patients with urinary tract infections.

Materials and Methods

Sample collection and identification: In this study, 3280 urine samples were collected from male (1430) and female (1850) patients with urinary tract infections in medical centers in Mazandaran Province, Iran from April to December 2022. First, sterile containers were given to the patients to collect urine samples, the first-morning urine samples were used for culture, and the patient had to clean the urine area with soap and water and pour the urine sample into the container for testing. In the next step, the sample was cultured on blood agar and eosin methylene blue (EMB) agar media, in such a way that the sterilized standard loop was inserted into the sample and placed on two media, and then the sample was incubated at 37 °C for 24 hours. After bacterial growth on blood agar, Gram staining was done to determine culture purity and colony morphology, and Gram-positive cocci were selected for additional tests. Identification

of all isolates was done using biochemical tests such as catalase, coagulase, growth on mannitol salt agar medium, and novobiocin resistance test. *S. saprophyticus* was identified by positive catalase, negative coagulase and/or DNase, novobiocin resistance, absence of hemolysis, and mannitol fermentation tests [9].

Storage of isolated strains: The purified strains were kept on a nutrient broth medium for inoculation. After sufficient growth of the strains, 50% glycerol was added, and the sample was kept in a freezer at -70 °C.

Antibiotic sensitivity test: Determination of antibiotic resistance was done by disc dif-

fusion method based on Clinical and Laboratory Standards Institute (CLSI, 2018) standards. To do so, microbial suspension equivalent to half McFarland was prepared on Mueller-Hinton agar medium, and antibiotic discs including erythromycin, azithromycin, and clarithromycin were placed on the desired medium. After 18 to 24 hours of incubation at 37 °C, the diameter of the growth halo around each disc was measured, and the results were recorded.

Out of 50 *S. saprophyticus* isolates, only 42 isolates had the necessary standards to perform the test. The criterion for determining the resistance level was based on CLSI stan-

Table 1) Primer sequences of all selected genes in the present study

Gene Name	Primers(5' →3')	Amplicon Size(bp)	Temperatures (° C)	Time	Cycle
<i>gyrA</i> -F <i>gyrA</i> -R	CGAGTGAGATGCGCGAGTCATTCTT ACGTTGACGACCGCCACCAC	731	94	4 min	1
			94	30 s	
			54	1 min	30
			72	1min	
			72	8 min	1
<i>parC</i> -F <i>parC</i> -R	ACGTTTCGTGATGGGCTCAAACCT ACGTAATCCAGTACGGTCTGTCTCA	797	94	4 min	1
			94	30 s	
			60	40 s	30
			72	1 min	
			72	8 min	1
<i>blaZ</i> -F <i>blaZ</i> -R	ATGTAATTCAAACAGTTCACATGC ATAGGTTTCAGATTGGCCCTTAGG	702	94	4 min	1
			94	30 s	
			60	45 s	30
			72	1 min	
			72	8 min	1
<i>ermC</i> -F <i>ermC</i> -R	GCTAATATTGTTTAAATCGTCAATTCC GGTCAGGAAAAGGACATTTTAC	573	94	4 min	1
			94	30 s	
			60	40 s	35
			72	1 min	
			72	5 min	1
<i>ermB</i> -F <i>ermB</i> -R	CCGTTTACGAAATTGGAACAGGTAAA GAATCGAGACTTGAGTGTGC	360	94	4 min	1
			94	40 s	
			58	40 s	35
			72	1 min	
			72	5 min	1
<i>ermA</i> -F <i>ermA</i> -R	TCTAAAAAGCATGTAAAAAGAAA CGATACTTTTTGTAGTCCTTC	533	94	30 s	1
			94	1 min	
			59	40 s	35
			72	40 s	
			72	5 min	1

dards. Accordingly, for erythromycin, halo diameter ≥ 23 mm was considered as sensitive, 14-22 mm as intermediate, and ≤ 12 mm as resistant. For azithromycin, halo diameter ≥ 18 mm was considered as sensitive, 14-17 mm as semi-sensitive, and ≤ 13 mm as resistant. For clarithromycin, halo diameter ≥ 21 mm was considered as sensitive, 17-20 mm as semi-sensitive, and ≤ 16 mm as resistant.

DNA extraction: To extract DNA of *S. saprophyticus* from colonies confirmed by biochemical methods, a DNA extraction kit from Gram-positive bacteria (Takapost Company) was used. The extracted DNA samples were kept in a freezer at -20 °C for the next stages of testing and Polymerase Chain Reaction (PCR). Plasmid DNA extraction was done using a Takapost plasmid DNA extraction kit according to the company's protocol.

Qualitative and Quantitative measurements of the extracted genome: To ensure DNA extraction and check the quality of the extracted plasmid DNA, 5 μ L of the solution containing the plasmid was mixed with 1 μ L of the loading solution, and electrophoresis was performed on 0.8% agarose gel. Electrophoresis was done horizontally using TAE (EDTA-acetate-Tris) buffer and 100 V voltage. After staining with ethidium bromide (0.5 μ g/mL), the agarose gel was examined with a gel dock device.

Primer selection: To perform PCR, primers capable of amplifying *gyrA*, *parC*, *blaZ*, *ermC*, *ermB*, and *ermA* genes were used. The primers were synthesized by Rubin Teb and prepared according to the manufacturer's instructions ^[4].

Searching for specific genes *gyrA*, *parC*, and *BlaZ*: In this research, specific primers for *gyrA*, *parC*, *blaZ*, *ernC*, *ernB*, and *ernA* genes were used to identify these genes. We designed the specific primers using Oligo 7 Primer Analysis Software. The sequences of the relevant primers and the thermal program used in PCR reactions are listed in Table 1.

Preparing (master mix) PCR Mix and performing PCR reactions: PCR was performed with 25 μ L of reaction mixture containing 3 μ L of DNA, 12.5 μ L of Super PCR Master Mix 2x (Yekta Tajhiz Azma Co), 1 μ L of each primer (20 pm), and 7.5 μ L of de-ionized water. PCR was performed in a master-cycler gradient thermal cycler (Eppendorf 5331, Hamburg, Germany). PCR primers and annealing temperatures are listed in Table 1.

PCR product evaluation: After the completion of the PCR reaction, 10 μ L of the PCR product and 2 μ L of the loading buffer were poured into the gel wells to observe the fragments. To read the size of the desired bands, 2 μ L of 100 bp DNA ladder was placed in one

Table 2) Frequency and percentage of bacteria detected in urine samples of patients in the present study

Genus or Species	Number	Percent
<i>Streptococcus pyogenes</i>	25	1.19
<i>Staphylococcus epidermidis</i>	383	18.39
<i>Staphylococcus saprophyticus</i>	52	2.49
<i>Staphylococcus aureus</i>	15	0.71
<i>Klebsiella</i>	253	12.11
<i>Escherichia coli</i>	1153	55.22
<i>Citrobacter</i>	38	1.81
<i>Enterobacter</i>	48	2.29
<i>Proteus</i>	32	1.53
<i>Pseudomonas</i>	35	1.67
<i>Enterococcus</i>	8	0.38
Other	46	2.20
Total	2088	

of the wells. Electrophoresis was performed under 100 V conditions for 45 minutes. Then the gel was observed and photographed by a gel recorder.

Sequencing: To determine the sequence of the studied samples, one sample of the PCR product of each gene was sent to Rubin Teb Company, and the results were sent by the

company to check and compare the resulting synonyms with the gene synonyms available in the gene bank in Blast software. BLAST analysis of PCR products was confirmed with more than 90% identity. The PCR product confirmed by DNA sequencing and blasting in the GenBank database was used. In the PCR reaction mixture, distilled water was

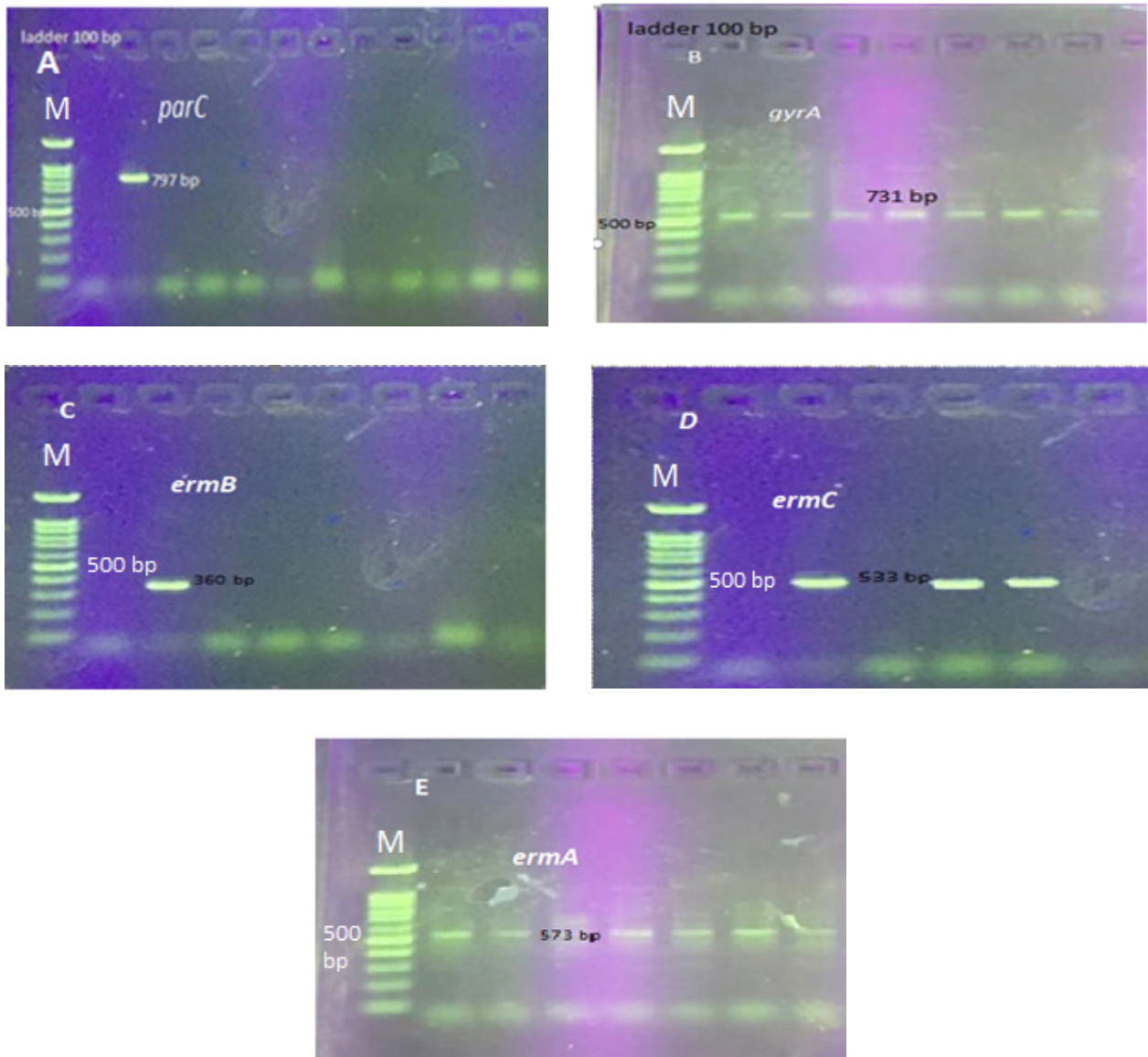


Figure 1) Agarose gel electrophoresis of PCR products of *parC* (A), *gyrA* (B), *ermB* (C), *ermC* (D), and *ermA* (E) genes in *Staphylococcus saprophyticus*. Lane M: size marker

In this study, 52 *S. saprophyticus* isolates were examined using PCR to detect *blaZ*, *gyrA*, and *parC* genes, 12 (23%) isolates harbored the *gyrA* gene, and one (1.92%) isolate harbored the *parC* gene. The *blaZ* gene was not observed in any of the samples, while one (1.92%) isolate harbored both *gyrA* and *parC* genes.

The prevalence of *ermA*, *ermB* and *ermC* resistance genes was 21% (n=11), 1.92% (n=1) and 26% (n=14), respectively.

used as a negative control.

Statistical evaluations: Experiment data drawing was done using Excel 2010 software, and if necessary, statistical analysis was done using SPSS software Ver.17.

Findings

Out of a total of 3280 urine samples collected, only 2088 samples were diagnosed as significant bacteria by microbial culture. Among the isolated bacteria, *Escherichia coli* (55.22%) and staphylococci (21.59%) were the most frequent bacterial isolates, respectively. A summary of the results of these tests is given in Table 2.

PCR test results

PCR products of the *gyrA* and *parC* genes produced fragments of 731 and 797 bp on agarose gel, respectively, but the *blaZ* gene did not. Gel electrophoresis of PCR products of *ermA*, *ermB*, and *ermC* genes produced fragments of 533, 360, and 573 bp, respectively (Figure 1).

The frequency of resistant, intermediate, and sensitive isolates to the studied antibiotics is shown in Figure 2. As could be observed, the highest antibiotic resistance of the isolates was related to erythromycin (70%), followed by azithromycin (36%) and clarithromycin (20%). On the other hand, the highest antibiotic sensitivity of the isolates was related to clarithromycin (46%).

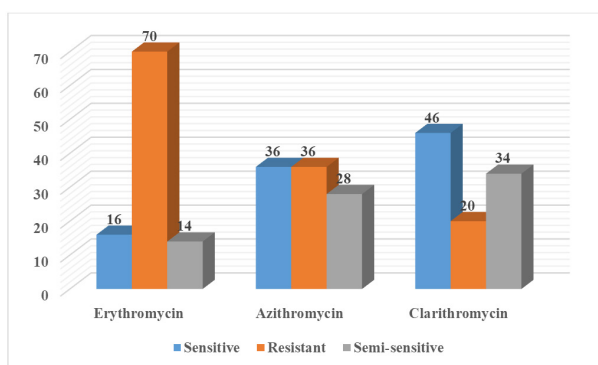


Figure 2) The Prevalence of Erythromycin, Azithromycin, and Clarithromycin Resistance among *Staphylococcus saprophyticus* isolates from urinary tract infections in Mazandaran Province, Iran

Discussion

Urinary tract infections (UTIs) take place due to the dominance of different bacteria over the body's defenses in the urinary tract. They could influence the bladder, kidneys, and the tubes that run between them. Thus, UTI is considered as the most common infection among infectious diseases, which has a significant financial burden from an economic point of view. Therefore, timely and correct diagnosis and treatment of this bacterial disease have a significant impact on its prognosis. Since resistance and sensitivity to antibiotics change with time and place factors, timely diagnosis and treatment of urinary infections are necessary, especially in cases involving the upper parts of the urinary system because delay in treatment causes irreversible damage [11].

Today, staphylococcal species that are prominent in medicine play a relatively important role in the etiology of urinary tract infections [5]. In this study, the molecular method showed that out of 52 *S. saprophyticus* isolates, 12 (23%) isolates harbored the *gyrA* gene, and one (1.9%) isolate harbored the *parC* gene. The *blaZ* gene was not observed in any of the samples. While one (1.92%) isolate harbored both *gyrA* and *parC*, 28 (70%) samples lacked the three studied genes. A study reported a 63.3% prevalence for the *gyrA* gene in coagulase-negative *Staphylococcus* isolates in three teaching hospitals in Hamedan [6]. Osman et al. (2017) in Egypt showed a prevalence of 63.3% for the *gyrA* gene in *Staphylococcus* isolates [7]. Another study showed the presence of *gyrA* and *parC* genes in 20 out of 21 *S. epidermidis* strains [5]. Studies have shown that more than 50% of *S. aureus* strains show mutations in *gyrA* [8]. In the present study, the *blaZ* gene was not found in any of the investigated samples, which is not consistent with other studies results. In a study, the frequency of this gene in *S. saprophyticus* isolates was reported to

be 59.49% [4]. Studies conducted on *blaZ* types and their frequency in coagulase-negative groups have shown a very high prevalence of this gene among different strains of *S. saprophyticus* and *S. epidermidis*, which is not consistent with the present study results [8]. Another investigation in Zahedan showed the presence of the *blaZ* gene in more than 90% of *S. saprophyticus* isolates, which indicates the high prevalence of beta-lactam resistant strains in this city [10]. Hisatsune et al. (2013) reported a prevalence of 98% for the *blaZ* gene [11], and Rosato et al. (2003) also obtained a 96% prevalence for this gene [12], which are not consistent with the present study results. Beta-lactamase production is a serious challenge in the treatment of infections caused by beta-lactamase producing organisms. Therefore, it is necessary to determine appropriate antibiotics and treatment approaches to control these infections using accurate and fast molecular tests [12, 13]. Most urinary tract infections are caused by microorganisms that enter the bladder outlet from the surrounding environment. The transmission of these infections is mostly through the urethra, in such a way that they reach the urethra through feces and ascend the urethra, which is more common in women than in men [14]. Urinary tract infections (UTIs) caused by antibiotic-resistant bacteria are one of the most common human bacterial infections among all age groups, which could have dangerous consequences if not treated in time and properly [15]. Urinary pathogens vary depending on age, gender, catheterization, hospitalization, and prior exposure to antibiotics [16]. The prevalence of antimicrobial resistance in urinary tract infections is increasing, and the amount of this resistance is different according to geographical and regional location [17]. Most urinary infections are superficial and do not cause serious damage. However, the importance of urinary infections is that if they are

diagnosed late or poorly treated, they cause complications that sometimes lead to kidney failure [18]. They may also cause irreparable damage and bacteremia in these people. In recent years, *Staphylococcus* has been prominent as one of the organisms causing infection in the hospital, and today the resistance of this bacterium to penicillin is increasing due to the production of beta-lactamase enzymes [19]. This organism has also become resistant to methicillin [20]. Among staphylococcal species, *S. saprophyticus* is the most common cause of urinary tract infection and in some reports, approximately 5-15% of urinary tract infections are caused by this bacterium [21-24]. According to the results of previous studies, the prevalence of coagulase-negative staphylococci in urinary tract infections is relatively high [25]. The results obtained from biochemical tests showed that 52 (2.49%) isolates were identified as *S. saprophyticus*. Also, the antibiotic sensitivity test results showed that 20, 36, and 70% of the isolates showed resistance to clarithromycin, azithromycin, and erythromycin antibiotics, respectively. In a study conducted by Hassanzadeh et al. (2021), 58% of *S. saprophyticus* strains isolated from urine were resistant to erythromycin, which is slightly different from the present study results [26].

Conclusions

In this study, the prevalence of resistance genes to erythromycin and fluoroquinolones in *S. saprophyticus* strains isolated from urinary tract infections was high; thus, prescribing these antibiotics should be done after checking the results of phenotypic or molecular resistance tests.

Acknowledgements

The authors express their gratitude and appreciation to all people who contributed to this manuscript.

Conflicts of interests: None declared by authors.

Ethical issues: There are no ethical issues for this article.

Authors' contributions: RA and AKhE wrote the manuscript and were involved in all parts of the project. ArA was involved in some experimental sections of the project. MA and RM edited and revised the manuscript technically and managed the project as a supervisor. All the authors confirmed the final edited version of the manuscript.

Consent to participate: Written informed consent was obtained from all patients.

Fundings: None declared.

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