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ORIGINAL ARTICLE

Sulfonamide resistance, virulence traits, and in-silico target interactions among clinical isolates in Setif, Algeria (2021–2023)

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ABSTRACT

Background and Objectives: Antibiotic-resistant bacteria are a growing global health concern, particularly in developing regions. Sulfonamides, once widely used, now face increasing resistance. This study assessed the prevalence, resistance profiles, and virulences traites of sulfonamide-resistant strains in Sétif, Algeria (2021-2023).

Materials and Methods: A total of 215 clinical isolates were collected from patients aged 1 day to 96 years (mean 42.7). Most were community-acquired (77.2%), with urinary tract infections predominating (49.3% in women, 32.1% in men). Identification and susceptibility testing followed standard microbiological and Kirby–Bauer methods. Virulence factors (biofilm, hemolysin, protease, lecithinase, and lipase) were examined. Molecular docking compared sulfamethoxazole and trimethoprim binding to their enzymatic targets.

Results: *Escherichia coli* was the most frequent isolate (47.9%), followed by *Enterobacter* spp. (11.6%). Biofilm formation was common (88.8%), with complete production in *Klebsiella*, *Citrobacter*, *Providencia*, and *Acinetobacter*. Hemolysis patterns were α (30.7%), β (27.9%), and none (41.4%). Enzymatic activity included protease (48.8%), lecithinase (22.8%), and lipase (9.8%). High resistance was observed to penicillins (87.9%), cephalosporins (63.7%), and fluoroquinolones (56.3%). Resistance was lower to imipenem (33.0%) and amikacin (14.4%). Docking showed weaker sulfamethoxazole binding to DHPS than trimethoprim to DHFR.

Conclusion: The high prevalence of multidrug-resistant bacteria, especially *E. coli*, combined with biofilm and enzyme production, underscores the urgent need for careful antibiotic stewardship in this region.

Keywords: Drug resistance; Sulfonamides; Anti-bacterial agents; Virulence factors; Molecular docking simulation

INTRODUCTION

Antibiotics are drugs widely used to treat bacterial infections. Since their discovery, they have saved millions of lives and have become essential in human and veterinary medicine (1). They are used to treat many common pathologies, as well as to prevent infections during medical procedures. However, their effectiveness depends on responsible and appropriate use, as improper use can lead to bacterial resistance (2).

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Antibiotic resistance is now a serious threat worldwide. According to a report published in 2022 by the World Health Organization (WHO) (3), around 5 million deaths in 2019 were associated with infections due to resistant bacteria, including 1.27 million directly caused by this resistance (4). Low- and middle-income countries are often the hardest hit, due to limited access to appropriate treatments and lack of control over antibiotic consumption. This phenomenon compromises the effectiveness of modern healthcare and necessitates a rapid, coordinated response.

Sulfonamides are among the historical antibiotics used in medicine. Their effectiveness was rapidly threatened by the emergence of resistance in several types of bacteria. These resistances have spread through different mechanisms, reducing the efficacy of sulfonamides in many cases (5).

The objective of this study was to evaluate the prevalence, virulence factors, and antibiotic resistance patterns of clinical bacterial strains resistant to sulfonamides in the Setif region, while assessing the molecular interactions of sulfonamide compounds with their bacterial targets through in silico docking analysis. This approach aimed to better understand the local epidemiological landscape of antimicrobial resistance and support the development of effective therapeutic strategies.

MATERIALS AND METHODS

This study was conducted in several geographical locations around Setif province in the northeastern of Algeria; the collection were distributed on seven medical diagnostic laboratories, two laboratories from the southwest of the province (Aïn Oulmene city), one from the extreme north (Bouandas city), two laboratories from the capital (Setif city), two laboratories from the north (Tizi N'Bechar city and Bougaa city) and three government hospitals: Ain Oulmene and El-Eulma hospitals, and Setif university hospital. The study was approved by the Ethics and Deontology Committee in University Ferhat Abbas of Setif-1- under the study registered with the number of the paper, UFAS1/09/03/2023/ETH-Deon-A-301, and an informed written consent was taken from each participant.

The study carried out between 2021 and 2023 on 215 bacterial strains isolated from various clinical samples, including mainly urine, blood, pus, vaginal swabs, bladder catheters. Samples were collected from both males and females of all age groups. A positive culture, free from contamination and respecting sterility criteria, showing decreasing sensitivity (total resistance to trimethoprim/sulfamethoxazole) was included in this study. Samples with incomplete clinical or laboratory data, those that did not meet quality control or sterility criteria, and those that did not adhere to proper temperature and duration of conservation before analysis, were excluded from this study.

Our samples were collected from two sources: community patients (outpatients) and hospitalized patients (inpatients admitted for treatment after 48 hours), representing different healthcare settings. All samples were handled under rigorous aseptic conditions. They were then inoculated onto a variety of culture media, both selective and non-selective including nutrient agar, hektoen agar, macConkey agar, Chapman agar and nutrient broth. Subsequently, they were incubated at 37°C for 24 hours.

Identification of bacterial strains. Bacterial strains were identified by microscopic examination, colony morphology analysis and gram staining. Biochemical tests were carried out, including indole production, fermentation of mannitol, glucose, sucrose and lactose on TSI agar, as well as catalase, oxidase, urease and gas production tests. This initial identification was confirmed by API 20^{E} galleries for enteric bacteria and API Staph for *S. aureus.* The strains identified were preserved in nutrient broth enriched with 30% sterile glycerol, enabling them to be preserved for subsequent analyses.

Antibiotic susceptibility testing. Bacterial strains were tested for antibiotic susceptibility using the Kirby-Bauer disk diffusion method and followed the EUCAST recommendations. Strains were grown on Mueller-Hinton agar (6), and bacterial inoculum was prepared from young cultures (18-24 h) in 0.9% NaCl saline, adjusted to the corresponding McFarland 0.5 density (OD = 0.08 – 0.13 at 625 nm, 10^{8} CFU/mL). This suspension was spread evenly over the surface of the dishes using a sterile swab, then disks impregnated with 24 antibiotics were carefully placed, including penicillins: amoxicillin (AX, 25 µg), ticarcillin (TC, 75 µg), and piperacillin (PRL, 100 µg), as well as β-lactam/ inhibitor combination: amoxicillin+ clavulanic acid (AMC, 30 µg), ticarcillin+ clavulanic acid (TTC, 85 µg), and piperacillin–tazobactam (TPZ,

110 µg), cephalosporin including cefalexin (CL, 30 µg) from the first, cefoxitin (FOX, 30 µg) from the second, cefixime (CFM, 5 µg), ceftazidime (CAZ, 30 µg), and cefotaxime (CTX, 30 µg) from the third, and cefepime (FEP, 30 µg) as a fourth-generation agent. Carbapenem: imipenem (IMP, 10 µg), aminoglycoside: gentamicin (CN, 10 µg), amikacin (AK, 30 µg), tobramycin (TOB, 10 µg), and kanamycin (K, 30 µg), quinolone and fluoroquinolone groups: nalidixic acid (NA, 30 µg), ciprofloxacin (CIP, 5 µg), ofloxacin (OFX, 5 µg), and levofloxacin (LEV, 5 µg), the phenicol chloramphenicol (C, 30 µg), the monobactam aztreonam (ATM, 30 µg), and the sulfonamide combination trimethoprim–sulfamethoxazole (SXT, 25 µg).

The antibiotics were selected according to species appropriate applicability. After incubation for 20 ± 4 h at 35 ± 2°C, the zones of inhibition were measured and compared with the critical diameters defined by EUCAST guideline (2022). Quality control strains, such as *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212, were used to classify strains as resistant (R), susceptible (S), or intermediate (I). Strains were classified as Multi-Drug Resistant (MDR), defined as resistance to at least one drug in three or more antibiotic classes, and Extensively Drug Resistant (XDR), defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories.

Detection of virulence factor production: biofilm. One of the virulence factors studied is the ability to form biofilm, which plays an important role in pathogenicity and a protective mechanism against antibiotics. Biofilm formation was demonstrated using the microplate method as described by O'Toole (2011) (7), Stepanović et al., (2007) (8), and Türkel et al. (2018) (9).

First the bacterial strains were grown on non-selective agar medium, then incubated overnight (24 hours) at 37°C, then the purity of the strains was checked, and a bacterial suspension was prepared in BHIB supplemented with 2% glucose and with a density of 0.5 McFarland. Subsequently, 200 µL were poured into polystyrene microtiter plates, 3 wells being used for each bacterial isolate, and incubated at 37°C for 24 hours. Following incubation, the medium was removed and the wells rinsed three times with distilled water to remove planktonic cells. 150 µL of methanol was added to each well, and left for 20 min to fix adherent cells. The biofilms were then stained with 0.2% (w/v) crystal violet solution for 15 min. Excess crystal violet was then removed; each well was washed with distilled water and the plates were exposed to air. 150 µL of 95% ethanol was used to solubilize the biofilms. Finally, absorbance was measured using an ELISA reader at 570 nm (BioTek, El Dorado Hills, CA, USA). Sterile broth was used as negative control, and *E. coli* ATCC 25922 was the control organism.

Based on optical density (OD), four levels of biofilm production were distinguished. Strains with an OD equal to or lower than that of the ODc were classified as non-biofilm producers. Those with an OD higher than the ODc but less than twice (2 ODc) were considered as low biofilm producers. Moderate biofilm production was defined when the OD was between two and four times that of the ODc. Finally, strains with an OD greater than four times the control were categorized as high biofilm producers.

Hemolysin. This method is based on the protocols of Mogrovejo-Arias et al., (2020) (10) and Tula et al., (2023) (11). Hemolysin production was assessed on agar enriched with 5% blood. Bacterial isolates were first cultured in BHIB. The culture medium was then prepared by incorporating 5% human blood into the agar base at a temperature of 45°C-50°C. The strains were then inoculated onto the agar and incubated at 37°C for 18-24 hours.

According to the appearance around the colonies, three types of hemolysis were distinguished. β-hemolysis, or complete hemolysis, was characterized by a clear zone around the colonies corresponding to total destruction of red blood cells. α-hemolysis, or partial hemolysis, produced a greenish halo around the colonies, which results from the oxidation of hemoglobin to methemoglobin. Finally, γ-hemolysis, or absence of hemolysis, was defined by the lack of visible change in the surrounding medium.

Protease production. Protease production is a key virulence factor which plays a major role in increasing the pathogenicity of microorganisms. To detect this activity, skim milk agar plates were prepared. The medium used contained peptone, yeast extract and agar, and was sterilized by autoclaving. Next, 100 mL/L of sterile UHT non-fat milk was added to the agar, and heated to 45-50°C. Bacterial strains were inoculated onto these plates, then incubated at 37°C for 24 hours. Proteolytic activity was revealed by the formation of a clear zone around the colonies, indi-

cating the presence of protease. *E. coli* ATCC 25922 was used as a negative control, while *P. aeruginosa* ATCC 27853 served as a positive control (12, 13).

Lecithinase and lipase production. To detect lecithinase and lipase production, strains were grown on egg yolk enriched agar (EYA), then incubated at 37°C for 24 hours. A nutrient medium was prepared by adding a sterile egg yolk emulsion to agar, heated to 45-50°C, to promote bacterial growth and enzyme production. The presence of lecithinase was identified by the appearance of a halo around the colonies, a sign of the enzyme's activity. *Bacillus cereus* and *E. coli* ATCC 25922 were used as positive and negative control. As for lipase, its production has been observed by the iridescent sheen that forms on the surface of colonies. Sometimes, to obtain clearer results, the plates were incubated for a period of 5 days to a week, at the same temperature. To check the validity of the tests, *P. aeruginosa* ATCC 27853 was used as a positive control, and *S. aureus* ATCC 29213 as a negative control (13).

Statistical analysis. The resistance data were analyzed using SPSS software (version 27). Frequencies of the categorical variables were calculated, and comparisons between percentages in different groups were performed using Pearson's Chi-squared test or Fisher's exact test when appropriate. A p-value ≤ 0.05 was considered statistically significant. The descriptive analysis focused on the distribution of resistance according to sex, age, age groups, and sampling sources, as well as on the prevalence of resistance by bacterial species, by virulence factor, and by antibiotic.

Molecular docking study. Molecular docking simulations were performed with AutoDock Vina 1.1.2 (AMdock 1.5.2) using two target enzymes: dihydropteroate synthase (PDB ID: 3TYB) and dihydrofolate reductase (PDB ID: 3SRW) (Fig. 1). The docking grids were centered on the native ligand coordinates to encompass the active sites, with dimensions of 30 × 30 × 30 Å for 3TYB (centered at -64, 60, 138) and 30 × 30 × 30 Å for 3SRW (centered at 7, -32, 6). Validation was carried out by redocking the co-crystallized ligands, yielding an RMSD value of 0.34 Å between the docked and crystallographic poses. As this value is well below the commonly accepted threshold of 2.5 Å, it confirms the reliability and robustness of our docking protocol (14). The reference ligands,

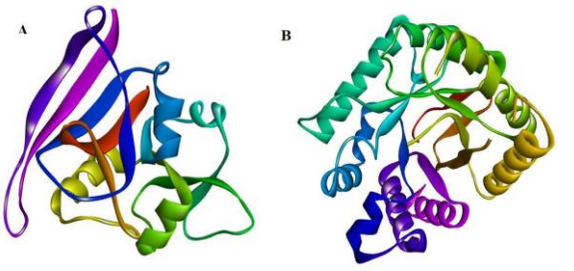

Fig. 1. 3D structure of the two proteins used, (a): Dihydrofolate Reductase (PDB ID: 3SRW) and (b): Dihydropteroate Synthase (PDB ID: 3TYB)

sulfamethoxazole and trimethoprim, were retrieved from PubChem and energy-minimized in Avogadro to obtain stable low-energy conformations. Protein structures were prepared in Discovery Studio by removing crystallographic waters not directly involved in ligand binding, and non-essential cofactors were removed, followed by the addition of polar hydrogens and Kollman charges (15). The processed ligands and proteins were subsequently used for molecular docking with AutoDock Vina.

Docking free energies (ΔG) were converted into inhibition constants using Ki = exp(ΔG/RT) (R is the gas constant (1.985 × 10-3 kcal mol−1 K-1) and T is temperature in kelvins=298.15 K). To validate the docking protocol, the co-crystallized inhibitors of each target enzyme were retrieved from the corresponding PDB entries and redocked into their binding sites. These ligands served as positive controls, comparing predicted and experimental binding poses. In addition, a decoy set of structurally similar but biologically inactive compounds was collected from the DUD-E database. The decoys were docked using the same parameters to evaluate the enrichment capacity of the docking workflow, ensuring that the protocol could reliably discriminate active molecules from inactive ones.

RESULTS

Prevalence of resistance by gender, age, age group and sampling source. Analysis of the data reveals a different distribution of age groups by gender. Adults make up the majority in both groups, with a greater proportion of women (75.4%) than men (57.3%). Children accounted for 22.5% of males vs 9.5% of females, while the elderly were relatively well distributed (20.2% among men and 15.1% among women).

The chi-square test shows a statistically significant association between gender and age group (p = 0.010) (Table 1).

Patient ages in the 215 collected samples ranged from 1 day to 96 years, with an average of 42.74 years, a median of 41.5 years, and an interquartile range (IQR: 25-75), indicating significant diversity within the study population.

In addition, the study of sample sources reveals a predominance of urine samples, mainly in women (49.3%), as opposed to men (32.1%). Some sources vary according to gender: for example, pus is more common in women (6.5% vs. 4.2%), while vaginal swabs are found exclusively in women (1.4%). More invasive specimens, such as catheters, blood or tracheal tubes, remain rare (less than 1.5%) and also show gender differences. The chi-square test reveals a statistically significant association between gender and source (p = 0.030).

Prevalence of resistance by bacterial species. In this study, most of the bacteria isolated belonged to the Gram-negative bacilli group, mainly *E. coli* (47.9% of the total), followed by *Enterobacter* spp. (11.63%). In contrast, bacteria identified in the Gram-positive group were exclusively Gram-positive cocci, such as *Staphylococcus* spp. (8.4%), *Streptococcus* spp. (1.9%) and *Enterococcus* spp. (0.9%).

There was a significant relationship between bacterial species and category, with a strong correlation, as shown by the chi-square test (p < 0.001). Of the 215 bacterial isolates identified, 166 (77.2%) came from the community, while 49 (22.8%) were of hospital origin. The most frequent species was *E. coli* with 103 isolates, of which 93 (90.3%) were community-acquired and 10 (9.7%) hospital-acquired. *Enterobacter* spp. Came second with 25 isolates, 16 of which were community-acquired and 9 hospital-acquired. Other common species include *Proteus* spp. (19 isolates, all community-acquired), *P. aeruginosa* (20 isolates, 11 community-acquired and 9 hospital-acquired), *Staphylococcus* spp. (18 isolates, 7 community-acquired and 11 hospital-acquired). Bacteria such as *Acinetobacter* spp. and *Enterococcus* spp. were found exclusively in hospital or community samples.

Detection of virulence factor production

Biofilm production. Of the 215 bacterial isolates, 191 (88.8%) expressed biofilm production, and only 24 isolates (11.2%) were non-producers. *E. coli* was the species most frequently responsible for 41.9% of biofilm cases, while 13 strains (6.0%) were not. Other species showing production included *Enterobacter* spp. (11.2%), *Proteus* spp. (8.4%) and *Staphylococcus* spp. (7.4%). Some species, such as *Klebsiella* spp. and *Citrobacter* spp., *Providencia* spp. and *Acinetobacter* spp. were found to be biofilmogenic in 100% of cases. In contrast, species such as *M. morganii* and *Enterococcus* spp. showed a balance between producing and non-producing strains (Table 2).

The Fig. 2 shows that the majority of *E. coli* strains exhibit low biofilm production (34.4%). Non-producing strains showed a lower proportion (6.1%), followed by moderate (6.5%) and high (1%) producers. This suggests that *E. coli* isolates rarely develop high intensity biofilms.

Hemolytic activity. 30.7% of strains were characterized by α-hemolysis, and 27.9% showed β-hemolysis (Table 3). The remaining strains showed no hemolysis. With regard to *E. coli* isolates, 22.3% were found to be γ-hemolytic, 8.8% showed α-hemolysis, while 16.7% showed β-hemolysis. Moderate levels of hemolysin activity were observed in *Proteus* spp., *Enterobacter* spp., and *Pseudomonas aeruginosa*, each showing mixed α- and β-hemolytic profiles. In contrast, *Klebsiella* spp., *Enterococcus* spp., and *Staphylococcus* spp. demonstrated low hemolysin expression, with a predominance of non-hemolytic isolates.

Lytic enzymes. The protease is the most frequently produced, with 48.8% of positive strains versus 51.2% non-producers. Lecithinase is less widespread:

Table 1. Distribution of age groups by gender.

			Tranche of age			
			Adult	**Children**	**Elderly**	**Total**
Gender	Male	Count (%)	51 (57.3)	20 (22.5)	18 (20.2)	89 (100)
	Female	Count (%)	95 (75.4)	12 (9.5)	19 (15.1)	126 (100)
Total		Count (%)	146 (67.9)	32 (14.9)	37 (17.2)	215 (100)

Table 2. Biofilm production among strains.

Species	Production Count/ (%)	Non production Count/ (%)	Total Count/ (%)
Enterobacter spp.	24 (11.2)	1 (0.5)	25 (11.6)
Proteus spp.	18 (8.4)	1 (0.5)	19 (11.6)
E. coli	90 (41.9)	13 (6.0)	103 (47.9)
Citrobacter spp.	7 (3.3)	0 (0.0)	7 (3.3)
P. aeruginosa	16 (7.4)	4 (1.9)	20 (9.3)
M. morganii	1 (0.5)	1 (0.5)	2 (0.9)
Staphylococcus spp.	16 (7.4)	2 (0.9)	18 (8.4)
Klebsiella spp.	10 (4.7)	0 (0.0)	10 (4.7)
Enterococcus spp.	1 (0.5)	1 (0.5)	2 (0.9)
Providencia spp.	4 (1.9)	0 (0.0)	4 (1.9)
Acinetobacter spp.	1 (0.5)	0 (0.0)	1 (0.5)
Streptococcus spp.	3 (1.4)	1 (0.5)	4 (1.9)
Total	191 (88.8)	24 (11.2)	215 (100)

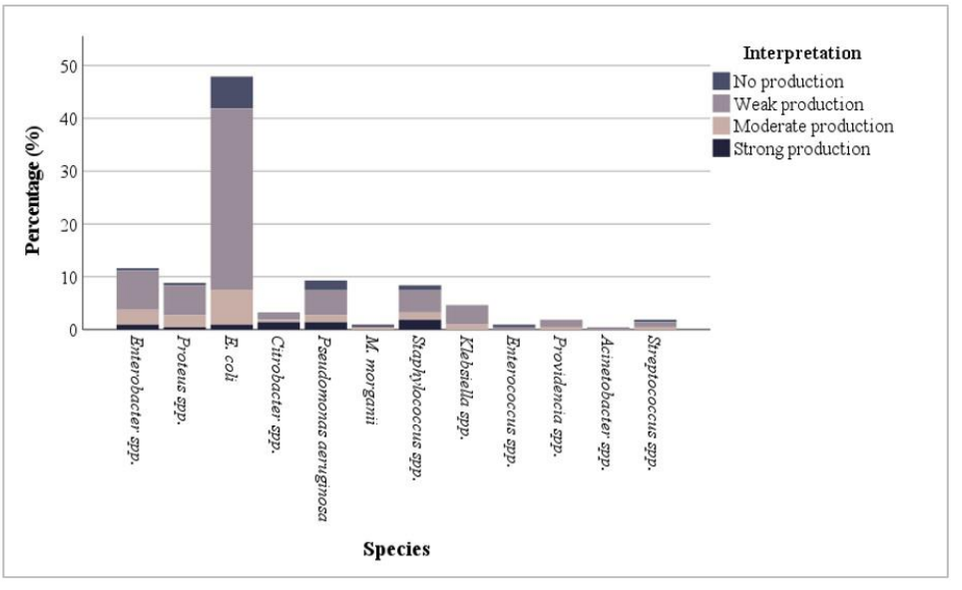

Fig. 2. Biofilm production level in species.

observed in 22.8% of strains, while 77.2% do not produce it. Finally, lipase is the least expressed enzyme, with only 9.8% of strains producing it, compared with 90.2% not producing it (Table 3).

Prevalence of resistance by antibiotic. Analysis of resistance profiles, carried out on strains resistant to trimethoprim-sulfamethoxazole, reveals a worrying situation. High resistance rates of 87.9% and 77. 7% were also observed for amoxicillin and amoxicillin/clavulanic acid, respectively. A 63.7% resistance rate was observed for first-generation cephalosporins, such as cephalexin, while resistance rates for fluoroquinolones, including ofloxacin and ciprofloxacin, were 56.3% and 47.4%, respectively. Conversely, Resistance remained relatively low for amikacin (14.4%), piperacillin–tazobactam (12.6%), and levofloxacin (5.6%) (Fig. 3).

Table 3. Enzyme production among trimethoprim-sulfamethoxazole resistant strains

Enzymes	Protease	Lecithinase	Lipase
Non producers (%)	51.2	77.2	90.2
Producers (%)	48.8	22.8	9.8

Molecular docking study. A computer-based molecular docking study was conducted to explore how sulfonamides interact with bacterial targets and to shed light on possible resistance mechanisms. The analysis focused on the binding of trimethoprim and sulfamethoxazole to their respective enzymes. The results are presented in terms of binding energy (kcal/mol) and estimated inhibition, as shown in Table 4.
The various interactions identified between trimethoprim and the active site residues are summarized in Fig. 4.

Trimethoprim showed a minimum binding energy of -7.6 kcal/mol in its most favorable pose, corresponding to an estimated Ki of 2.69 µM and a binding efficiency of -0.36. Other poses also demonstrated moderate to high affinities, with Ki values reaching up to 14.53 µM.

Docking analysis of trimethoprim with DHFR revealed multiple stabilizing interactions within the enzyme's active site. Classical hydrogen bonds with ALA8 and ILE15 residues ensured strong fixation, while additional carbon–hydrogen bonds with ASN1, SER5, and PHE9 contributed to secondary stabilization. π–Hydrogen donor bonds (ALA8, THR47) and an amide–π stacking interaction (ILE15) further reinforced orientation and surface binding. Moreover, hydrophobic (alkyl) and π–alkyl contacts with residues LEU21, LYS46, VAL7, and ALA8 promoted firm anchoring of the ligand, confirming the strong affinity and stability of the trimethoprim–DHFR complex.

The main interactions established between sulfamethoxazole and the protein active site residues (DHPS) are summarized in Fig. 5.

Sulfamethoxazole exhibited a minimum binding energy of -6.6 kcal/mol, corresponding to an estimated Ki of 14.53 µM and a binding efficiency of -0.39. The

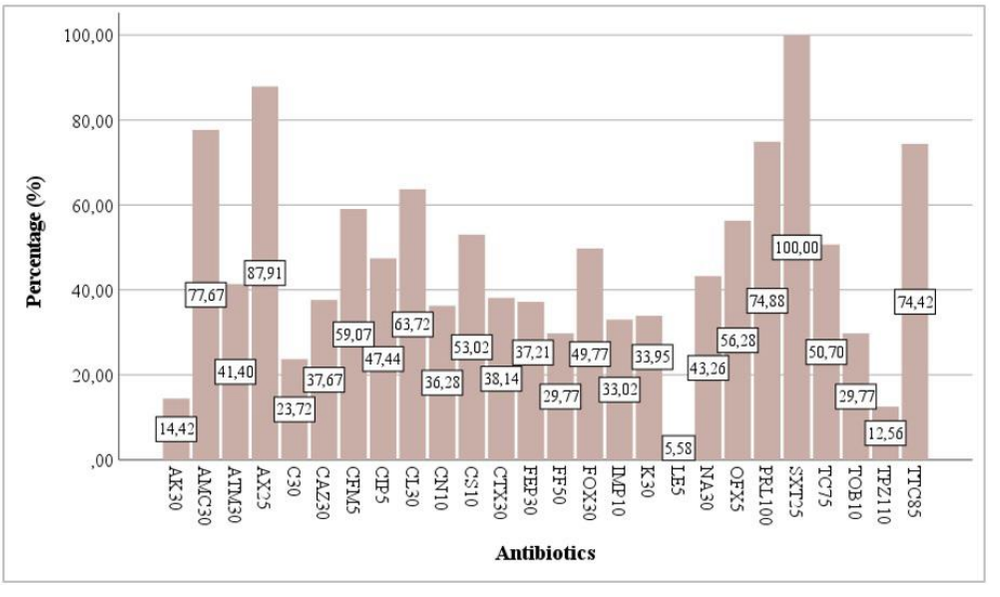

Fig. 3. Antibiotic resistance profile of trimethoprim + sulfamuthoxazole resistant strains (AK30: amikacin, AMC30: amoxicillin+clavulanic acid, ATM30: aztreonam, AX25: amoxicillin, C30: chloramphenicol, CAZ30: ceftazidime, CFM5: cefixime, CIP5: ciprofloxacin, CL30: cephalexin, CN10: gentamycin, CTX30: cefotaxime, FEP30: cefipime, FOX30: cefoxitin, IMP10: imipenem, K30: kanamycin, LEV5: levofloxacin, NA30: nalidixic acid, OFX5: ofloxacin, PRL100: piperacillin, SXT25: trimethoprim/sulfamethoxazole, TC75: ticarcillin, TOB10: tobramycin, TPZ110: piperacillin+tazobactam, TCC85: ticarcillin+clavulanic acid).

Table 4. Binding parameters of antibiotics with their targets.

Ligands	Proteins	Energy (kcal/mol)	Ki (µM)	Energy (kcal/mol) (Control positive)
Trimethoprim	3SRW (DHFR)	-7.6	2.69	-8.7
Sulfamethoxazole	3TYB (DHPS)	-6.6	14.53	-5.6

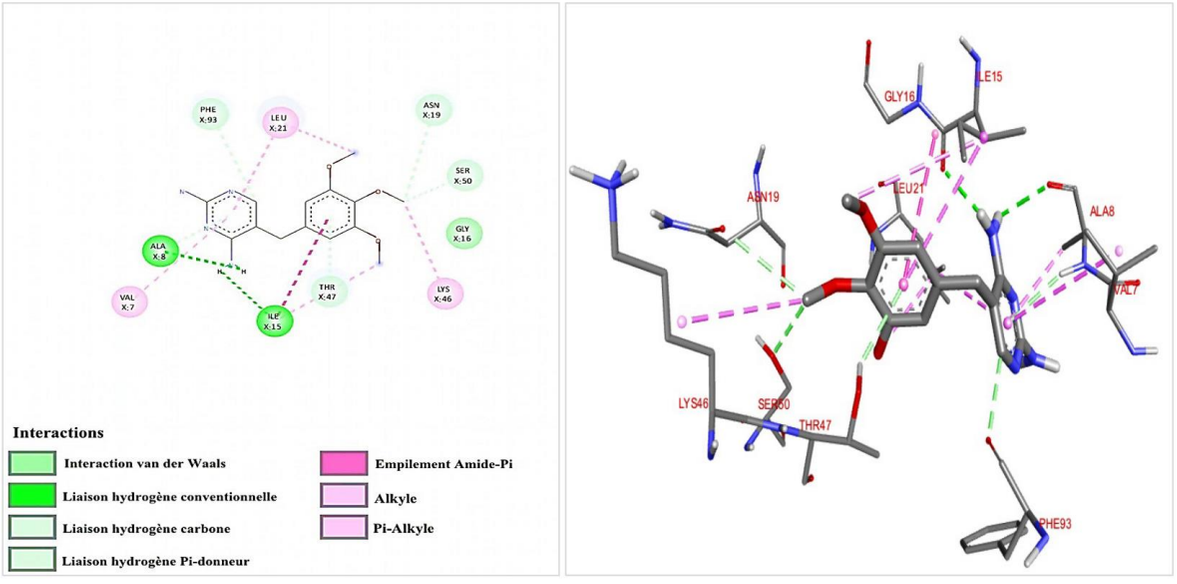

Fig. 4. Visualization of 2D and 3D binding sites of trimethoprim with DHFR.

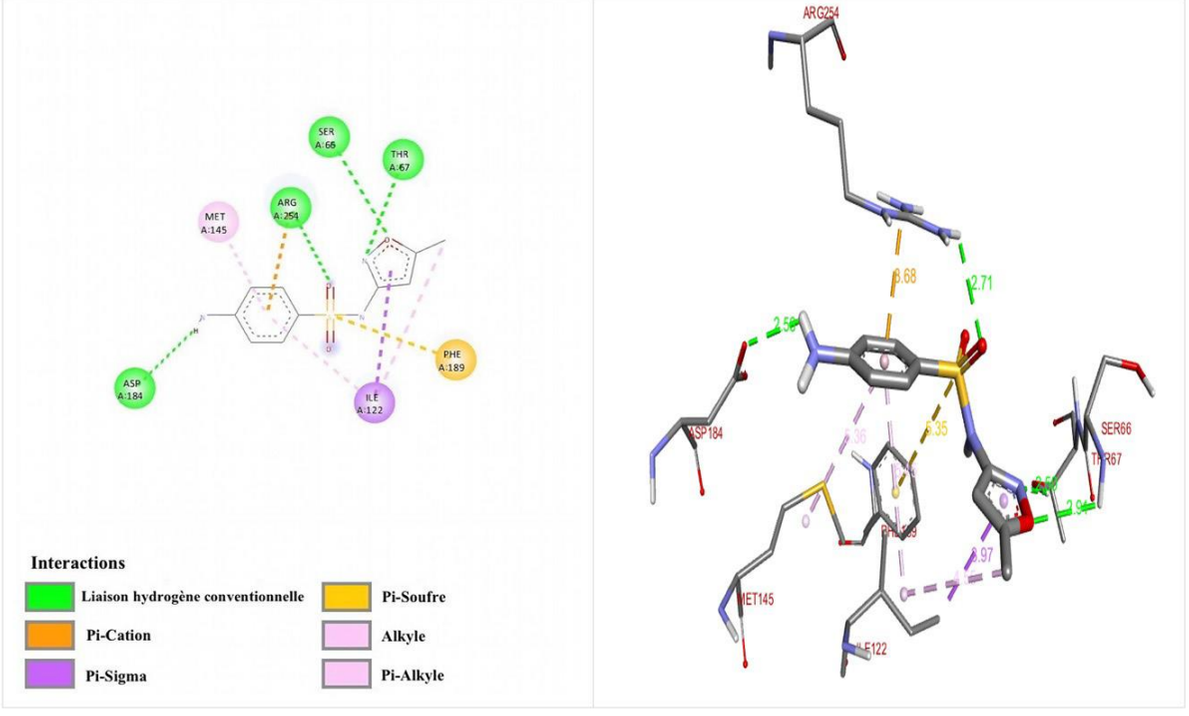

Fig. 5. Visualization of 2D and 3D binding sites of Sulfamethoxazole with DHPS.

Ki values of other binding poses ranged from 17.20 to 47.35 µM.

Molecular docking analysis revealed that sulfamethoxazole (NL1) interacts strongly with the active site of DHPS through multiple stabilizing forces. Classical hydrogen bonds involving SER66, THR67, ARG254, and ASP1 ensure strong fixation of the ligand via donor–acceptor interactions. An electrostatic attraction between ARG254 and the ligand's π-orbitals reinforces anchoring, while π–sigma (ILE122) and π–sulfur (PHE189) contacts contribute to complex stability. In addition, hydrophobic (alkyl) and π–alkyl interactions

with ILE122 and MET1 residues further enhance the ligand's retention within the binding pocket, confirming a strong affinity and stable conformation of sulfamethoxazole at the enzyme's active site.

Thus, although sulfamethoxazole showed a lower affinity for DHPS compared to trimethoprim's affinity for DHFR in our study, it is still strong enough to account for the effectiveness of co-trimoxazole. This synergistic combination of the two compounds works by sequentially inhibiting two key enzymes involved in the bacterial folate biosynthesis pathway.

DISCUSSION

This study reveals a clear predominance of adult patients, particularly women (75.4%), compared with men (57.3%). This trend may be linked to the increase in urinary tract infections (UTIs) among women. In this respect, a study carried out in Saudi Arabia by Alhazmi et al., (2023) revealed that women accounted for 62.66% of UTI cases (16). Similarly, Aslam et al., (2020) reported a higher incidence of UTIs in adult women (17), highlighting their increased vulnerability, particularly after the menopause. This susceptibility is mainly explained by anatomical and hormonal factors. From an anatomical point of view, the female urethra, shorter and located closer to the anus, facilitates the ascent of bacteria to the bladder. From a hormonal point of view, fluctuations in estrogen levels, particularly after the menopause, can disrupt the protective vaginal flora, thus increasing the risk of UTIs, as shown by Czajkowski et al., (2021) (18). This trend is further supported by the higher frequency of urine samples taken in women, underlining the trend towards higher prevalence of UTIs in women. This finding is in line with the results of other studies, such as those by Nzalie et al., (2016) and Kwok et al., (2022), who attributed this predisposition to anatomical and pathophysiological factors specific to women, such as the shorter urethra and sexual activity (19, 20).

In men, although the prevalence of UTIs is lower, the presence of a group of elderly men (20.2% of the male sample) is notable, as this age group is more likely to develop risk factors for UTIs, such as benign prostatic hyperplasia. For example, one study showed that in older men, the most important patho-mechanism of UTI is benign prostatic hyperplasia (BPH) (21).

Another study carried out in three Arab countries showed that women accounted for 68.45% of UTIs cases among 168 patients, compared with 31.55% among men. The majority of female cases involved women of childbearing age (14-44 years), while male cases mainly affected elderly men (52-85 years) (22).

Furthermore, in the pediatric population, the higher proportion of UTIs in boys (22.5%) compared to girls (9.5%) could be explained by differences in exposure or immune response to pathogens at a young age. According to Tullus and Shaikh's (2020) study of UTIs in children, boys, especially uncircumcised infants, are at increased risk of UTIs due to specific anatomical and microbial factors (23). This observation supports the idea that biological and anatomical factors could contribute to the higher prevalence observed in boys in our study.

In the elderly, the relatively even gender distribution (20.2% in men and 15.1% in women) suggests that age is a major risk factor in the occurrence of UTIs, probably linked to age-related immune alterations. Indeed, according to Rodriguez-Mañas (2020), the phenomenon of immune-senescence, examples: the gradual decline in the immune response with age, increases the susceptibility of older adults, whatever their sex, to UTIs, particularly when associated with other factors such as comorbidities or exposure to nosocomial pathogens (24). This heightened vulnerability could explain the almost symmetrical gender distribution in the geriatric population. As for the slight male predominance observed, this has already been discussed above.

Concerning the source of samples, the predominance of urinary samples in women (49.30%) compared to men (32.09%) is consistent with numerous studies reporting a higher incidence of UTIs in women (19, 20). Female-specific risk factors, such as shorter urethra and sexual activity, are often cited (25).

The results obtained are consistent with several previous studies. For example, Mohsenzadeh et al., (2020) reported that 88.1% of bacterial isolates in their study were Gram-negative bacilli, a proportion similar to that in our study (88.8%) (26). What's more, *E. coli* was also the most prevalent bacterium in their population (56.7%), compared with 47.9% in our data, confirming its major role in community-acquired UTIs. Similarly, Kiiru et al., (2023) reported that *E. coli* was the most common pathogen in patients without major complications (27).

The exclusive presence of Gram-positive cocci such as *Staphylococcus* spp. may indicate a nosocomial origin or a compromised terrain, such as catheter use, comorbidities or immunosuppression. This is in line with the findings of Raoofi et al., (2023) who associated these bacteria with opportunistic infections in hospitalized or vulnerable patients (28). The importance of *Enterobacter* spp. and *P. aeruginosa* in hospital samples may be linked to nosocomial factors: invasive medical devices, prolonged antibiotic therapy or comorbidities, as mentioned by Svebrant et al., (2021). Finally, the variation in species according to source (community vs. Hospital) confirms that certain bacteria are more associated with community infections (29).

Through analysis of the results, 88.8% highlights the key role of biofilm in UTIs, serving as a protective barrier against antibiotics and a major virulence factor (30). This polymeric matrix helps the bacteria to resist and survive in the urinary tract by providing protection from hydrodynamic forces, immune defenses, phagocytosis, as well as antibiotics (31).

There was a higher rate than that reported by Rajmane et al., (2021) in an Indian study where only 47% of 352 urinary isolates showed biofilm production (32). This difference can be explained by various factors, including variations in hygiene levels, geographical differences, study duration, increasing antibiotic resistance, and above all by the diversity of sample isolation sources (urine, pus, catheter, etc.) (31).

These results are in line with those reported by Arafa et al., (2022), where 1000 urinary *E. coli* was described as weakly biofilmogenic. The authors of this study attribute this phenomenon to their community origin, which implies less exposure to hospital environments that favor the expression of virulence factors such as biofilm formation (33). The Chi-square test ($p = 0.050$) indicated that there was a significant association between this low biofilm production and the community origin of *E. coli*, confirming the results obtained, this is consistent with the study by Hiremath and Lava (2020), who showed that *E. coli* is hemolytic in 35.5% of cases. Type A hemolysin (HlyA) produced by uropathogenic *E. coli* is actively involved in severe UTIs (34). In high doses, this hemolytic toxin destroys cells, enabling them to cross tissue barriers and gain access to nutrients. At low doses, it induces an apoptotic process in immune and epithelial cells, promoting their exfoliation (35). Furthermore, a study by Ristow and Welch (2016) showed a correlation between hemolysin production and severe forms of UTI, indicating its severity in UTIs (36).

The data reveal a percentage of lecithinase production comparable to observations made by Razzaq et al., (2022), where 13% of aquatic strains showed lecithinase activity (37). This concordance suggests that lecithinase production in aquatic environments is relatively low but present. This modest rate could be attributed to the genetic specificity of the strains isolated, as well as to the nature of the medium used for enzyme induction. Some strains only produce this type of enzyme under optimal environmental conditions or in the presence of specific substrates.

Conversely, protease production in our study was significantly higher. This contrasts sharply with the results of Razzaq et al., (2022), who observed only one strain (4%) producing this enzyme (37). This significant variation can be explained by several factors: the diversity of bacterial species present, the physico-chemical conditions of the medium of origin, and the detection methods used. For lipase, only 9.77% of isolates expressed lipolytic activity, suggesting a relatively low capacity for production of this enzyme under the experimental conditions. This trend is in line with the results reported by Abdelaziz et al., (2025), who detected lipase production in 28.3% of the isolates studied. The low percentage of production could be linked to environmental induction (38). Lipase production is often stimulated by the presence of lipid substrates in the environment. In addition, inter-species differences and intra-species genetic variations could account for the observed variability.

Our antibiotic resistance data are similar to those reported by Benmoumou et al., (2023) in their research carried out in Algiers, focusing on enterobacteria strains recovered from community-acquired urinary tract infections. These authors also reported high rates of penicillin resistance, with 91%, in agreement with our data. Conversely, only 33.62% were resistant to cotrimoxazole, lower than in our study, which could be explained by local differences in prescribing habits or in the frequency of patients' prior exposure to this molecule (39).

It's important to point out that the study by Benmoumou et al., (2023) was limited to *Enterobacteriaceae*, whereas our work covers a wider range of species than *Enterobacteriaceae*, including non-*Enterobacteriaceae* species such as *P. aeruginosa*, *En-*

terococcus spp. and *Staphylococcus* spp. (39).

This microbial variability could be at the root of the variations observed in resistance profiles. For *Enterococcus* spp. a recent multicenter study in Algeria revealed the presence of *Enterococcus faecium* isolates resistant to vancomycin (VRE), all classified in the vanA genotype, and presenting multi resistance to at least five classes of antibiotics (40). A study carried out in Cameroon also showed that all *Staphylococcus* spp. isolates were resistant to cefazolin and cefixime, with 76.2% showing multidrug resistance (41).

Docking results indicate a strong affinity of trimethoprim for the active site of the dihydrofolate reductase (DHFR) enzyme, suggesting a stable and potentially inhibitory interaction. This finding aligns with previous studies, which have showed that trimethoprim binds effectively to bacterial DHFR, forming key hydrogen bonds with residues such as Asp27, Ile5, Ile94 (42). Our findings are consistent with several recent studies. For instance, one study reported that sulfonamide derivatives interact with key residues in the active site of DHPS-namely Arg63, His257, and Lys221 with binding energies as low as -10.75 kcal/mol. This supports the idea that these inhibitors effectively target the enzyme (43).

CONCLUSION

Our study established the epidemiological and phenotypic profile of trimethoprim-sulfamethoxazole–resistant bacterial strains isolated in Setif from 215 infected patients. The results revealed that adult women were the most affected group, particularly in community settings, where urinary tract infections predominated among the analyzed samples. *Enterobacteriaceae*, especially *Escherichia coli*, were the main species responsible for resistant community-acquired urinary tract infections. Regarding virulence, a considerable proportion of strains exhibited biofilm-forming capacity, although most were of low productivity, particularly in *E. coli*. Hemolytic activity was detected in only a few isolates, while the production of enzymes such as protease, lecithinase, and lipase was generally low to moderate. Despite the moderate virulence observed, multidrug resistance was frequent, especially to β-lactams. Nevertheless, some antibiotics including amikacin, piperacillin–tazobactam, and levofloxacin remained effective against virulent strains, with levofloxacin emerging as the most active in several cases.

The molecular docking analysis showed relatively low binding energy values, reflecting significant efficacy of these antibiotics toward their targets. However, this efficacy may be reduced in vivo due to mechanisms such as mutations altering target protein conformation, decreased membrane permeability, or activation of efflux pumps. Taken together, the in silico results support the experimental findings and highlight the value of integrating molecular docking with biological data to better elucidate bacterial resistance mechanisms.

It is important to emphasize that the molecular docking analysis in this work was not intended to introduce novel mechanistic insights into the action of co-trimoxazole, whose targets (DHFR and DHPS) are already well established. Instead, docking was employed as a supportive tool to illustrate the discrepancy between predicted drug–target affinity and the high prevalence of resistance observed in our isolates. While docking confirmed the expected binding of trimethoprim and sulfamethoxazole to their respective enzymes, the persistence of resistance in clinical strains, especially *E. coli*, underscores the contribution of additional mechanisms such as efflux pumps, reduced membrane permeability, and biofilm-mediated tolerance. In this way, the docking results bridge structural predictions with clinical microbiology outcomes, reinforcing that target interactions alone cannot explain antimicrobial resistance in our region. These findings, while promising, should be interpreted as preliminary, as clinical outcomes and MIC distributions were not assessed. They may serve as a basis for future studies exploring the relationship between molecular docking results, phenotypic resistance, and clinical response. Overall, our work provides valuable epidemiological and molecular data that may inform antimicrobial surveillance efforts and generate hypotheses for future research on treatment optimization and resistance mitigation strategies.

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