Research Article

Studying the Antibiotics Resistance Pattern in Pathogenic Strains of *Escherichia coli* and *Staphylococcus aureus* in District Sargodha, Punjab, Pakistan

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Abstract | Antibiotics are the chemicals that inhibit or destroy microbes, especially bacteria. Antibiotic resistance is a process by which bacteria become able to grow even in the presence of antibiotics. This study identifies the effectiveness of mostly prescribed antibiotics against *Escherichia coli* and *Staphylococcus aureus* in district Sargodha, Pakistan. A survey among doctors identified the ciprofloxacin, levofloxacin, co-amoxiclav, cefixime, azithromycin, amoxicillin and cefadroxil as the most commonly prescribed antibiotics in Sargodha. Bacterial isolates from the sample were tested through well diffusion assay and then miniumim inhibitory concentration (MIC), minimum bactericidal concentration (MBC) of these antibiotics were identified. Ciprofloxacin and co-amoxiclav were effective against both *E. coli* and *S. aureus* with MIC values of 8.3mg/ml, 10.7mg/ml for *E. coli* and 8mg/ml, 12.5mg/ml for *S. aureus* while cefixime (13.3 mg/ml), cefadroxil (14 mg/ml) and azithromycin (16 mg/ml) showed effectiveness against *E. coli* only. *E. coli* showed resistance to amoxicillin, while *S. aureus* was resistant to several mostly prescribed antibiotics including cefixime, amoxicillin, cefadroxil and azithromycin. The study highlights the need for the region-specific antibiotics policies due to rising resistance.

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Introduction

The term "antibiotics" originates from the French word "antibiose" or "antibiotique", as defined by Vuillemin in the late 19th century a substance that exerts harmful effects on the living organisms, particularly microorganisms (Halawa *et al.*, 2024). In 1947, it was defined later by Selman A. Waksman as

"chemical substances produced by microorganisms that kill or destroy other microbes", have become very important agents against bacterial diseases (Sodhi and Singh, 2022). The first antibiotic was penicillin that is a natural compound produced by microorganism, *Penicillium notatum* culture (Kovalakova *et al*., 2020).

Antibiotics fall into main categories according to their chemical nature and according to their spectrum of activity, as narrow-and broad-spectrum antibiotics (Yang *et al*., 2021). β-lactam antibiotics represent an important antibiotics group which prevents bacterial cells from building their cell walls for survival (Uddin *et al*., 2021). Fluoroquinolone is a group of synthetic antibiotics that have broad-spectrum activity against Gram-negative and a few Gram-positive organisms such as ciprofloxacin, levofloxacin. They exert their action by inhibiting two bacterial enzymes DNA gyrase and topoisomerase IV involved in the important processes of DNA replication, transcription, repair, and recombination (Uddin *et al*., 2021). Macrolide antibiotics are one of the prominent groups which inhibit the bacterial synthesis of protein through interference with the large ribosomal 50S subunit. Such as erythromycin, azithromycin, and clarithromycin (Parnham *et al*., 2014). The cephalosporins are a broad class of β-lactam antibiotics that are used for different bacterial infections (Stocco *et al*., 2020). Cephalosporins are a group of β-lactam antibiotics that interfere with the synthesis of the cell wall of the bacterial cell (Stafylis *et al*., 2021). Tetracyclines are a group of antibiotics that act by inhibiting bacterial protein synthesis-through binding with the 30S ribosomal subunit, they stop aminoacyl-tRNA from attaching to the ribosome (Uddin *et al*., 2021). Sulfonamides represent synthetic antibiotic compounds that prevent bacterial folic acid synthesis through antimetabolite actions (Uddin *et al*., 2021).

E. coli are the rod shaped gram-negative bacterial microorganisms within the Enterobacteriaceae family (Bonten *et al*., 2021). They were first isolated from infant stool and characterized by Theodor Escherich in 1885 (Pakbin *et al*., 2021). *E. coli* resides within human guts and leads to several types of infections such as those that affect the intestines as well as parts outside the gut (Bonten *et al*., 2021). *S. aureus* is a gram-positive spherical shaped bacterium that is a commensal pathogen belonging to the family Staphylococcaceae (Lakhundi & Zhang, 2018). As a commensal bacterium *S. aureus* exists on human skin and mucous membranes but can still induce various infections from mild skin infections to severe bloodstream infections and endocarditis, osteomyelitis, skin and soft tissue infections (Tong *et al*., 2015).

Bacteria develop resistance to antibiotics by several mechanisms. One of the key strategies includes lowering the intracellular concentration of antibiotics. This can take place either due to reduced drug uptake, chiefly through alternation of porin channels or active efflux pumps located in the bacterial cell membrane (Darby *et al*., 2023; Halawa *et al*., 2024). Another mechanism of antibiotic resistance refers to the modification of the target site of the antibiotic (Shree *et al*., 2023). Bacteria can alter the structures of critical elements, including ribosomal subunits 30S or 50S, DNA gyrase, topoisomerase IV, penicillin-binding proteins (PBPs) (Hirsch & Klostermeier, 2021; Sodhi & Singh, 2022). Apart from limiting the accumulation of drugs inside cells and modifying targets, bacteria can also inactivate antibiotics by enzymatic means. For instance, beta-lactamase enzymes act to hydrolyze the beta-lactam ring in penicillins and cephalosporins to inhibit their activity (Fernández-Billón *et al*., 2023). The main aim of this study was to check which of the most commonly prescribed antibiotics in district Sargodha are effective against *E. coli* and *S. aureus* while against which of mostly prescribed antibiotics bacteria show resistance.

Methodology

This study was conducted by performing the following procedure

Data collection

A survey was conducted to collect the data about the most commonly prescribed antibiotics from the consultant doctors in District Sargodha. This research survey consisted of closed-ended questionnaire.

Isolation

For the isolation of bacteria from samples such as from blood or sputum, streak plate method was performed. This method involved streaking using inoculating loop and culturing bacteria on a nutrient agar plate at 37°C for about 24 hours. As a result, separate colonies of bacteria were maintained which were later identified by chemical tests (Sanders, 2012).

Identification

After the isolation by streak plate method, bacteria were identified by the gram staining and catalase test. Gram staining involves staining bacteria with primary dye such as crystal violet which is then treated with iodine to form iodine crystal violet complex. After that decolorize with absolute ethanol. And finally stained with safranin (secondary dye). Gram positive bacteria (*Staphylococcus aureus*) retained the crystal violet while gram negative bacteria (*Escherichia coli*) retained safranin (Paray *et al.*, 2023). In catalase test, hydrogen peroxide (H_2O_2) reacted with catalase enzyme in bacteria and break down into water and hydrogen as a result bubbling occurred which shows positive results (Ismail *et al.*, 2018).

Well diffusion assay

To assess which of the commonly prescribed antibiotics are effective against *E. coli* and *S. aureus* well diffusion assay was performed. This process involved spreading the bacterial sample on a nutrient agar plate. After that, wells were formed in agar plate by metal cork borer which are then filled with antibiotics. The plates were incubated at 37°C for 24 hours. After 24 hours zones of inhibition were formed around wells for some antibiotics which showed effectiveness. While wells with no zone of inhibition shows resistance (Chen *et al.*, 2019).

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration is the concentration of antibiotics at which growth of bacteria stops. Broth dilution method was used to find the MIC of mostly prescribed antibiotics. This involves culturing bacteria in diluted solution of antibiotics in nutrient broth at 37°C for 18 to 24 hours in incubator. After 24 hours samples were checked, cloudiness showed the growth of bacteria while clear solution showed no growth (Turlej-Rogacka *et al.*, 2018).

Minimum bactericidal concentration (MBC)

Minimum bactericidal concentration is the concentration of antibiotics that kills 99.9% of bacteria. This occurred after finding MIC and involved culturing bacteria taken from the broth dilution sample on antibiotics free agar plate at 37°C for 24 hrs in incubator. If growth occurred after 24 hrs it means that bacteria show resistance to antibiotics (Parvekar *et al.*, 2020).

Results

A research survey was performed to collect data about mostly prescribed antibiotics from 40 to 45 consultant doctors in district Sargodha Punjab Pakistan. As we know there are many antibiotics but after this survey it was found that the mostly prescribed antibiotics in district Sargodha are ciprofloxacin, levofloxacin, cefadroxil, co-amoxiclav, cefixime, amoxicillin and azithromycin (Figure 1). These antibiotics are available and prescribed in both doses such as 500mg and 250mg for ciprofloxacin, levofloxacin, cefadroxil, amoxicillin and azithromycin while 400mg and 200mg for cefixime, 625mg and 375mg for co-amoxiclav.

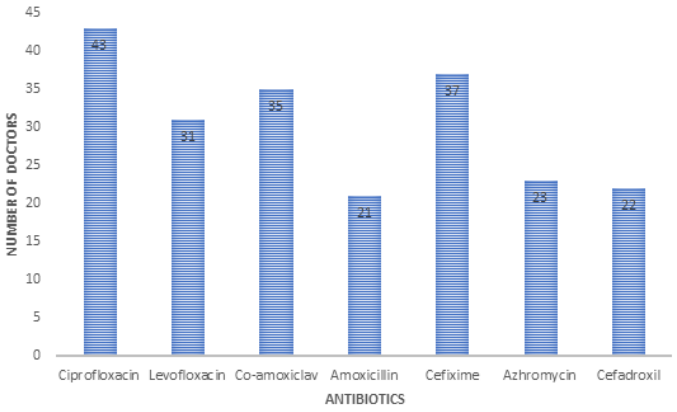

Figure 1: *Commonly prescribed antibiotics by the medical consultants in District Sargodha*

Escherichia coli and *S. aureus* are the bacteria that were used for this research study. These bacteria were separated from the sample by using a streak plate method. It involves culturing a sample on a nutrient agar plate at 37°C for 24 hrs. As a result, separate colonies of bacteria were produced. These bacteria were then identified by gram staining and catalase test. *E. coli* is gram negative bacteria appeared pink red while *S. aureus* being gram positive appeared purple. Both *E. coli* and *S. aureus* gave positive result in catalase test. In both case bubbling occurs in catalase test due to presence of catalase enzyme in both bacteria.

Antibiotics susceptibility was checked by performing well diffusion assay. This assay helps to identify which of most commonly prescribed antibiotics are effective and against which antibiotics bacteria develop resistance. After 24 hrs of culturing the bacteria along with antibiotics in wells of agar plate at 37°C zones of inhibition were produced. These zones were measured to check antibiotics effectiveness. Statistical analysis like mean and standard deviation were find by using

SPSS software. And the significant value was set at P≤0.05. Well diffusion assay was performed for both high and low doses of mostly prescribed antibiotics in district Sargodha (Table 1 & Table 2).

Table 1: *Zone of inhibition (cm) for escherichia coli and staphylococcus aureus at the highest dose rates of some commonly prescribed antibiotics*

Antibiotics	*Escherichia coli*	*Staphylococcus aureus*
Ciprofloxacin 500mg	1.6±0.15	1.4±0.12
Co-amoxiclav 625mg	1.8±0.14	1.5±0.12
Cefixime 400mg	1.1±0.70	1.3±0.12
Cefadroxil 500mg	1.6±0.12	NIL
Levofloxacin 500mg	0.3±0.54	NIL
Amoxicillin 500mg	NIL	0.7±0.42
Azithromycin 500mg	1.4±0.12	NIL

Table 2: *Zone of inhibition (cm) for escherichia coli and staphylococcus aureus at the lowest dose rates of some commonly prescribed antibiotics*

Antibiotics	*Escherichia coli*	*Staphylococcus aureus*
Ciprofloxacin 250mg	0.9±0.56	1±0.64
Co-amoxiclav 375mg	1.2±0.12	1.3±0.14
Cefixime 200mg	0.8±050	NIL
Levofloxacin 250mg	NIL	NIL
Amoxicillin 250mg	NIL	NIL
Azithromycin 250mg	NIL	NIL

After that minimum inhibitory concentration (MIC) of mostly prescribed antibiotics in district Sargodha was checked by performing broth dilution method. This method helped to identify which dose of mostly prescribed antibiotics are effective and against which *E. coli* and *S. aureus* develop resistance. After culturing the bacteria in nutrient broth along with antibiotics for 24hrs at 37°C MIC was identified (Table 3). Then minimum bactericidal concentration (MBC) of antibiotics was checked by culturing the bacteria taken from antibiotics treated nutrient broth solution on nutrient agar plates at 37°C (Table 3).

This research study has helped to identify that which of the most commonly prescribed antibiotics in district Sargodha are effective against *E. coli* and *S. aureus* while against which of these commonly prescribed antibiotics these bacteria develop resistance. Some of the mostly prescribed antibiotics were found effective at both high and low doses while against some *E. coli* and *S. aureus* develop resistance (Table 4 and Table 5).

Discussion

The main purpose of this research study was to check which of mostly prescribed antibiotics are effective against *E. coli and S. aureus* while against which antibiotics these bacteria develop resistance in district Sargodha. For this purpose, survey was performed to collect data about the mostly prescribed antibiotics (Figure 1). As the *E. coli* is gram negative bacteria it retained the secondary dye and appeared pink red while *S. aureus* is gram positive retained the primary dye and appeared purple during this research experiment. Both of these bacteria had given positive result for the catalase test also.

Antibiotic susceptibility was checked by well diffusion assay. From the previous studies it was found that ciprofloxacin had highest zone of inhibition for *E. coli* and *S. aureus* with diameter of about 3.7cm and 3.5cm which shows that ciprofloxacin has high antibacterial activity against these bacteria (Akani *et al.*, 2020). From the previous studies it was found that zone of inhibition of co-amoxiclav for *S. aureus* was 2 cm and for *E. coli* 2.3cm (Alhusayni & AL-Khikani, 2023; Momoh & Olaleye, 2022). Cefixime was also effective against *E. coli* and *S.s aureus* with the mean zone of inhibition equal to 3.4cm and 4cm (Mirghani *et al.*, 2018). Zone of inhibition for the well diffusion assay of cefadroxil for *E. coli* and *S. aureus* were 1.9cm and 1.8cm (Joenoes, 2021). Levofloxacin inhibitory zones for *E. coli* was 0.8cm and for *S. aureus* it was 1cm (Mohammad *et al.*, 2020; for *E. coli* and *S. aureus* were recorded at 2.5cm and 0.7cm (Jabir *et al.*, 2018). *S. aureus* gave no zone of Thakral *et al.*, 2023).

Table 3: *Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)*

	CIP	LVF	CAM	CFX	AMX	AZT	CFD
Escherichia coli	8.3mg/ml	14.2mg/ml	10.7mg/ml	13.3mg/ml	NI	16mg/ml	14mg/ml
Staphylococcus aureus	8mg/ml	16mg/ml	12.5mg/ml	NI	NI	NI	NI

CIP = ciprofloxacin, CFD = cefadroxil, CAM = co-amoxiclav, CFX = cefixime, LVF = levofloxacin, AMX=amoxicillin, AZT = azithromycin, NI = no impact

Inhibition zones of amoxicillin inhibition to azithromycin while *E.coli* gave about 2.1cm (Oliseloke *et al*., 2022). In this study Table 1 and Table 2 shows about values of the zone of inhibition of different antibiotics against *E. coli* and *S. aureus* at high and low doses. In our study, mean zones of inhibition of different antibiotics against *E. coli* are as ciprofloxacin (1.6-0.9cm), co-amoxiclav (1.8-1.2cm), cefixime (1.1-0.8cm), cefadroxil (1.6cm), levofloxacin (0.3cm), amoxicillin (NIL), azithromycin (1.4cm) (Table 1 & 2). While mean zones of inhibition for *S. aureus* are ciprofloxacin (1.4-1cm), co-amoxiclav (1.5-1.3cm), cefixime (1.3cm), cefadroxil (NIL), levofloxacin (NIL), amoxicillin (0.7cm), azithromycin (NIL) (Table 1& 2). By comparing with the previous it was found that some of the antibiotics prescribed in Sargodha have no impact on *E. coli* and *S. aureus*.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of mostly prescribed antibiotics was determined by broth dilution method and agar plates in this study. The past study showed that the minimum inhibitory concentration of ciprofloxacin for *E. coli* and *S. aureus* was about 0.3µg/ml and 0.02µg/ml (Sana *et al*., 2018). Levofloxacin MIC values from the previous study for the *E. coli* and *S. aureus* were found to be about 7.91µg/ml and 32µg/ml (Abdulabbas *et al*., 2022; Flamm *et al*., 2017). Co-amoxiclav inhibited the growth of *E. coli* and *S. aureus* at the minimum inhibitory concentration of about 15.63mg/ml and 7.81mg/ml (Momoh & Olaleye, 2022). The MIC values of cefixime from the previous studies for *E. coli* and *S. aureus* were about 125µg/ml and 150µg/ml (Asadi *et al*., 2023; Umekar *et al*., 2021). From the previous study, it was found that the amoxicillin has the minimum inhibitory concentration of about 2mg/ml for both the *E. coli* and *S. aureus*. While the MIC of cefadroxil for the *E. coli* and *S. aureus* was 4mg/ml and 1mg/ml (Purwanggana *et al*., 2018). The minimum inhibitory concentration of azithromycin was in the range of 0.625–2.5mg/ml for the *E. coli* while for the *S. aureus* it was found to be about 31.25mg/ml (Momoh *et al*., 2023; Taha, 2021). In this study Table 3 shows data about the MIC and MBC of available high and low dose of mostly prescribed antibiotics in the district Sargodha. In our research study it was found that minimum inhibitory concentrations of mostly prescribed antibiotics against *E. coli* are as ciprofloxacin (8.3mg/ml), levofloxacin (14.2mg/ml), co-amoxiclav (10.7mg/ml), cefixime (13.3mg/ml), amoxicillin (NI), azithromycin (16mg/ml), cefadroxil (14mg/ml). For *S. aureus* MIC values were 8mg/ml for ciprofloxacin, 16mg/ml for levofloxacin, 12.5mg/ml for co-amoxiclav, while there was no impact of cefixime, amoxicillin, azithromycin and cefadroxil. This research study has helped to identify which of mostly prescribed antibiotics were effective against *E. coli* and *S. aureus,* while against which of the available doses of these antibiotics *E. coli* and *S. aureus* developed resistance (Table 4& Table 5).

Conclusions and Recommendations

From this study, it was found that some of the most commonly prescribed antibiotics in district Sargodha, Punjab, Pakistan, were effective at both high and low doses against both the *E. coli* and *S. aureus*. While

Table 4: *Antibiotic sensitivity and resistance categories based on high dose of some most prescribed antibiotics in District Sargodha*

Bacterial strains	CIP 500mg	CFD 500mg	CAM 625mg	CFX 400mg	LVF 500mg	AMX 500mg	AZT 500mg
Escherichia coli	S	S	S	S	S	R	S
Staphylococcus aureus	S	R	S	R	S	R	R

CIP = ciprofloxacin, CFD = cefadroxil, CAM = co-amoxiclav, CFX = cefixime, LVF = levofloxacin, AMX=amoxicillin, AZT = azithromycin, S= Sensitivity, R= Resistance

Table 5: *Antibiotics sensitivity and resistance at low dose of mostly prescribed antibiotics*

Bacterial strains	CIP 250mg	CAM 375mg	CFX 200mg	LVF 250mg	AMX 250mg	AZT 250mg
Escherichia coli	S	S	R	R	R	R
Staphylococcus aureus	S	S	R	R	R	R

CIP = ciprofloxacin, CAM = co-amoxiclav, CFX = cefixime, LVF = levofloxacin, AMX=amoxicillin, AZT=azithromycin, S= Sensitivity, R= Resistance

these bacteria develop resistance against some of these antibiotics. Ciprofloxacin, levofloxacin and co-amoxicillin were found effective against both of these bacteria. Cefixime, cefadroxil and azithromycin were effective against *E. coli* only. While *E. coli* developed resistance only against amoxicillin out of these mostly prescribed antibiotics. But *S. aureus* developed resistance against cefixime, amoxicillin, cefadroxil and azithromycin. This study highlights the need for proper region-specific antibiotics profiling due to rising resistance trends.

Novelty Statement

This research study will be helpful in the proper use of antibiotics to reduce the chances of the antibiotics resistance and helps in the policy making regarding the use of the antibiotics.

Author's Contribution

Hammad Ahmad: Principal and project investigator, write-up, laboratory activities and corresponding author
Farzana Shahin: Supervised the research and helped in proofreading
Faiza Zubair: Helped in laboratory operations and research work
Muhammad Mudassar Maqbool: Helped in proof-reading and editing
Muhammad Shahid Nisar: Helped in data analysis
Sanaullah Yasin: Review and data compilation
Ahmad Kamran Khan: Proofreading and editing the manuscript.

Generative AI or AI assisted technology statement
The authors declare that no generative AI or AI assisted technology was used in the writing or editing of this manuscript.

Conflict of interest
The authors have no conflict of interest.

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