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ANTIMICROBIAL ACTIVITY OF RESIDUAL ANTIMICROBIALS IN HUMAN URINE AGAINST CLINICAL AND ENVIRONMENTAL ISOLATES

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ABSTRACT
The extensive use of antibiotics in human treatments is leading to rapid spread of antibiotic resistant bacteria, which poses high health risks to humans. This study aimed to determine the susceptibility of Gram-negative bacteria isolated from clinical and environmental sources to antimicrobial residues in urine of patients treated with antibiotics. A total of 10 urine samples were collected aseptically from inpatients under antibiotic treatments and were used to prepare sensitivity discs. The susceptibility of the clinical and environmental isolates to the residual antimicrobials in the urine was carried out using disc diffusion and broth dilution methods. The urine samples were diluted to various level and minimum inhibitory concentrations were determined. Results showed that 8 out of the 10 urine samples inhibited the growth of 59% of clinical isolates and 41% of environmental isolates with zones of inhibition ranging from 9.0 to 37.0 mm. Proteus mirabilis, and Klebsiella pneumoniae from clinical samples were susceptible to 7 out of the 10 urine samples. In addition, Pseudomonas aeruginosa from environmental sources is resistant to all but 1 urine sample. All the isolates were resistant to urine sample of patients undergoing treatments with Azithromycin. Four fold dilution of the urine was able to inhibit the growth of environmental and some clinical isolates. The results indicated urine samples of patients if not properly disposed, can result in evolution of antibiotic resistance in clinical and environmental bacteria.
Keyword: Antibiotics, Susceptibility, Clinical Isolates, Environmental Isolates Proteus mirabilis Klebsiella pneumoniae, Pseudomonas aeruginosa

INTRODUCTION

Antimicrobials are compounds administered to both humans and livestock for therapeutic and non-therapeutic purposes, such as disease prophylaxis and growth promotion (Daiana *et al.*, 2020; Yusuf *et al.*, 2019). Frequent use of these antimicrobials even when it is appropriately used to fight bacterial infection, puts pressure on bacteria to select for resistance and increase antimicrobial resistance (AMR) burden. Reducing unnecessary antibiotic use against viral infections and their rational use in human and animals have been shown to slow the spread of AMR (CDDEF, 2017; O'Neill, 2015; Tang *et al.*, 2017; Serrano *et al.*, 2021). Ideally, disease management would involve administering antibiotics to patients after an accurate diagnosis of the causal agent is done, through the collection of an accurate sample for bacterial culture and antimicrobial susceptibility testing.

After administration, a proportion of antibiotics or their metabolites are absorbed into various cells, tissues, and organs of the body, while other pharmaceutically active parts are excreted in form of antibiotic residues in urine and other wastes. The residual antimicrobials in the urine can then contaminate the environment and pose an ecological risk for the evolution of resistant bacteria that might threaten public health. In developing countries where open urination and defecation is common, urine could reach different environment media directly or indirectly through runoff and other waste water.

Poor sanitation, hygiene, and mismanagement of antibiotics in and outside hospitals, and other irrational use (for instance in agriculture) can lead to release of higher residues in urine. Specifically, around 40–90 % of administered antibiotics are excreted through urine and feces in active forms, which can contaminate the environment. In addition to that, accidental spillage of patient's urine on the floor of hospital wards and/or illegal disposal of contents of urine bags into the hospital toilet floors is very common. Some of the antimicrobials that might be present in urine such as erythromycin, sulfamethoxazole, and tetracyclines can persist in soil and water for a period of more than a year (Cycon *et al.*, 2019). Subsequently the evolved resistant bacteria can be transmitted to other human and animals through direct and indirect contact. In Nigerian health care centers and private settings a wide range of antibiotics are used to treat minor infections and major infections, but data on amount of antimicrobial residues in patient urines and their effect on clinical and environmental isolates are scarce.

Due to the growing concern over AMR in Nigeria and in its efforts to increase data on the burden of AMR and antimicrobial use (AMU) across all the sectors of one health in Nigeria, a study was designed to evaluate the antimicrobial susceptibility pattern of clinical and environmental isolates to the antimicrobial residues present in urine of patients admitted and treated for different diseases in Kano, Nigeria.

MATERIALS AND METHODS

Study Area and sample population

The study was conducted in Kano state Nigeria. The study population comprises of in-patients admitted into different wards of Barewa Clinic and Maternity for

different clinical manifestations. Ethical approval was approved by the clinic. The hospital is a private hospital located at Zaria Road, Kano State. It was established in 1995 and operates on 24hrs basis for 7 days. Samples of urine and demographic data were collected from 10 patients after securing their consents.

Inclusion Criteria

Consenting in-patients undergoing treatment at Barewa Clinic and Maternity at the time of sample collection.

Exclusion Criteria

Non-consenting patients and/ or patients not receiving antibiotics while on admission.

Demographic and clinical information of study participants

A structured questionnaire designed, tested and validated was used to collect the demographic and clinical information of the 10 consented patients. Sex, age and address were provided by the study participants, while clinical information were provided by clinician and nurses on behalf of the patients.

Urine Sample Collection

Non-repetitive Midstream clean catch urine samples were collected in a sterile, transparent round container, from inpatients receiving treatments with metronidazole, ciprofloxacin, azithromycin and or ceftriaxone in the hospital according to Cheesbrough (2010) procedures. Samples were transported immediately to the Microbiology Laboratory of Bayero University, Kano for use as source of antibiotics to prepare sensitivity discs

Isolation of bacteria from clinical and environmental samples

Environmental isolates already isolated from ecological soil by other colleagues in the team were collected from our collection centers. The identities of the isolates were reconfirmed by carrying out Gram staining and biochemical tests to identify their morphology, Gram reaction and biochemical characteristics as described by Cheesbrough (2010). Similarly, already isolated clinical isolates (*Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*) were collected from the Microbiology unit of Fadi Medical Laboratory (FML), Kano and Aminu Kano teaching hospital Kano (AKTH) and transported to Microbiology Laboratory Bayero University Kano in a Ziploc bag.

Determination of Susceptibility of the isolates to Urine residual antimicrobials

Single colonies of each clinical and environmental isolate were emulsified in sterile normal saline to make suspension of isolates. The suspension was adjusted to 0.5% McFarland standard as described by Cheesbrough (2010). A sterile cotton swab was dipped into the standardized bacterial suspension and evenly streaked onto Mueller-Hinton Agar plates. Meanwhile, a 6mm blank Whatmann No.1 filter paper was punched and sterilized in bottles in an autoclave. One hundred sterile 6mm discs were immersed in 1 milliliter of urine sediment obtained by centrifugation at 12,000g for 10 minutes and placed in bottles. The discs were allowed to absorb the urine, and excess urine was removed, then discs were allowed to dry. The discs were labeled based on the antibiotics the patient was receiving at the time of collection. The plates were incubated at 37^{o}C for 24 hours aerobically. The zone diameters were measured using vernier caliper in accordance with CLSI 2020 guidelines.

Determination of Minimum Inhibitory Concentration (MIC) of the urine

The minimum inhibitory concentration determination of the antimicrobial residues in the urine samples was carried out using broth dilution method. A nutrient broth was prepared according to manufacturer's guideline. All attempts to quantify the amount of residual antimicrobials in each urine using HPLC proved abortive, so an alternative dilution method was employed. A prepared nutrient broth was placed in a water bath to prevent it from solidifying before used. Six test tubes containing 9ml of sterile peptone water were serially arranged and labelled 100, 50, 25, 12.5%, negative control, and positive control. Concentrations of urine residues were diluted in folds in the test tube containing peptone water to obtain the concentration of 100, 50, and 25, of 12.5%. The initial 100% concentration was obtained from the original urine samples, and then diluted by 2, 4 and 8 folds to obtain 50, 25, 12.5% concentrations. One millilitre of each prepared bacterial strain was inoculated into the tubes with different concentrations of urine residues. A tube of peptone water supplemented with different test urine residues was left uninoculated and used as a negative control for each dilution. For positive control, 1ml of the bacterial strains were inoculated into a peptone water tube without the urine residues. Tubes were incubated aerobically at 37°C for 24 hours and the tube that shows no turbidity was considered as inhibiting bacterial growth. The lowest concentration that inhibits bacterial growth was considered as the MIC value for each of the tested bacteria strains (Hamisu *et al.*, 2019).

RESULTS

Analysis of demographic and clinical data collected shows that out of the 10 in-patients, 4 patients were receiving metronidazole, 3 receiving ceftriaxone, 2 receiving ciprofloxacin and 1 is on azithromycin treatment (Table 1). the age range of the study participants is 4-95 years, with mean age of 47.8 years. The clinical manifestations of the participants that spread over medical (40%), surgical (10%), amenity (20%), labour (20%), and post natal (10%) wards include caesarian section, eclampsia, chronic arm ulcer, sepsis and urinary tract infections (UTI). Fifty percent of the patients are receiving the antimicrobials orally while the other 50% are receiving theirs intravenously. The length of stay in the hospital showed that patients admitted into labour wards (20%) only spent less than 24 hours receiving ceftriaxone intravenously, while the longest stayed patient (20 days) was the one with chronic arm ulcer admitted into the Amenity ward.

Table 1: Demographic and clinical manifestation and types of antibiotics used by patients

Patient ID	Age	Sex	Clinical manifestation	Wards	Antibiotic treatment	Dosage	Mode of administration	Length of stay on admission
P1	22	F	Severe Preeclampsia	Post natal	Metronidazole	250mg	Oral	1day
P2	AD	F	Sepsis	Medical	Metronidazole	250mg	Oral	1day
P3	37	M	Leg ulcer	Medical	Metronidazole	500mg	Oral	Hours
P4	85	M	Prostate cancer	Medical	Metronidazole	500mg	Oral	3 days
P5	20	F	Eclampsia	Labour	Ceftriaxone	1g	IV	Hours
P6	30	F	Cesarian section	Labour	Ceftriaxone	1g	IV	Hours
P7	90	M	Chronic arm ulcer	Amenity	Ceftriaxone	1g	IV	20 days
P8	95	F	Sepsis	Amenity	Ciprofloxacin	500mg	Oral	3 days
P9	AD	F	UTI	Medical	Ciprofloxacin	500mg	Oral	4 days
P10	4	M	Tonsillectomy	Surgical	Azithromycin	5ml	Oral	7 days

The antimicrobial susceptibility testing of residual antimicrobials in urine against the isolates revealed that, *P. mirabilis* and *K. pneumoniae* from clinical sources are highly susceptible to urine samples of patients receiving ceftriaxone, but *E. coli* was resistant to the samples. *E. coli* from the environmental sources is highly susceptible to antimicrobial residues in almost all the urine samples, while the *E. coli* from the clinical sources are resistant to about 80% of the urines. Urine of patient receiving Metronidazole and Ciprofloxacin exhibit varying activities against all the tested clinical isolates, while urine of patients on azithromycin treatment showed no activity against all the tested clinical isolates. The highest zone of inhibition (36.52mm) was recorded by urine obtained from P9 against *P. aeruginosa* strain obtained from environmental source (Fig. 1). It is interesting to note that, all the isolates tested against urine sample of patient (P9) are highly susceptible, except *E. coli* and *K. pneumoniae* from clinical sources which produced zone of inhibition of 13.76mm and 9.41mm respectively. Urine residues of patients on ciprofloxacin are active against all tested environmental bacteria, while the urine of patients on azithromycin showed no antimicrobial activity against all the tested environmental bacterial isolates (Table 2).

Table 2: Zones of inhibition (mm) of urinary pathogen and bacteria from the soil to antimicrobial residues in human urine:

Patient ID	Clinical isolates			Environmental isolates		
	E. coli	*K. pneumoniae*	*P. mirabilis*	*E. coli*	*Shigella sp*	*P. aeruginosa*
P1	0	22.13	23.56	13.35	0	0
P2	0	25.76	24.95	18.81	0	0
P3	0	0	0	0	0	0
P4	20.16	25.56	16.64	19.39	17.72	0
P5	0	24.56	27.33	21.81	11.35	0
P6	0	23.25	27.33	21.06	0	0
P7	0	26.89	28.88	25.54	0	0
P8	22.28	18.97	9.04	24.5	17.62	0
P9	13.76	9.41	23.01	23.4	29.31	36.52
P10	0	0	0	0	0	0

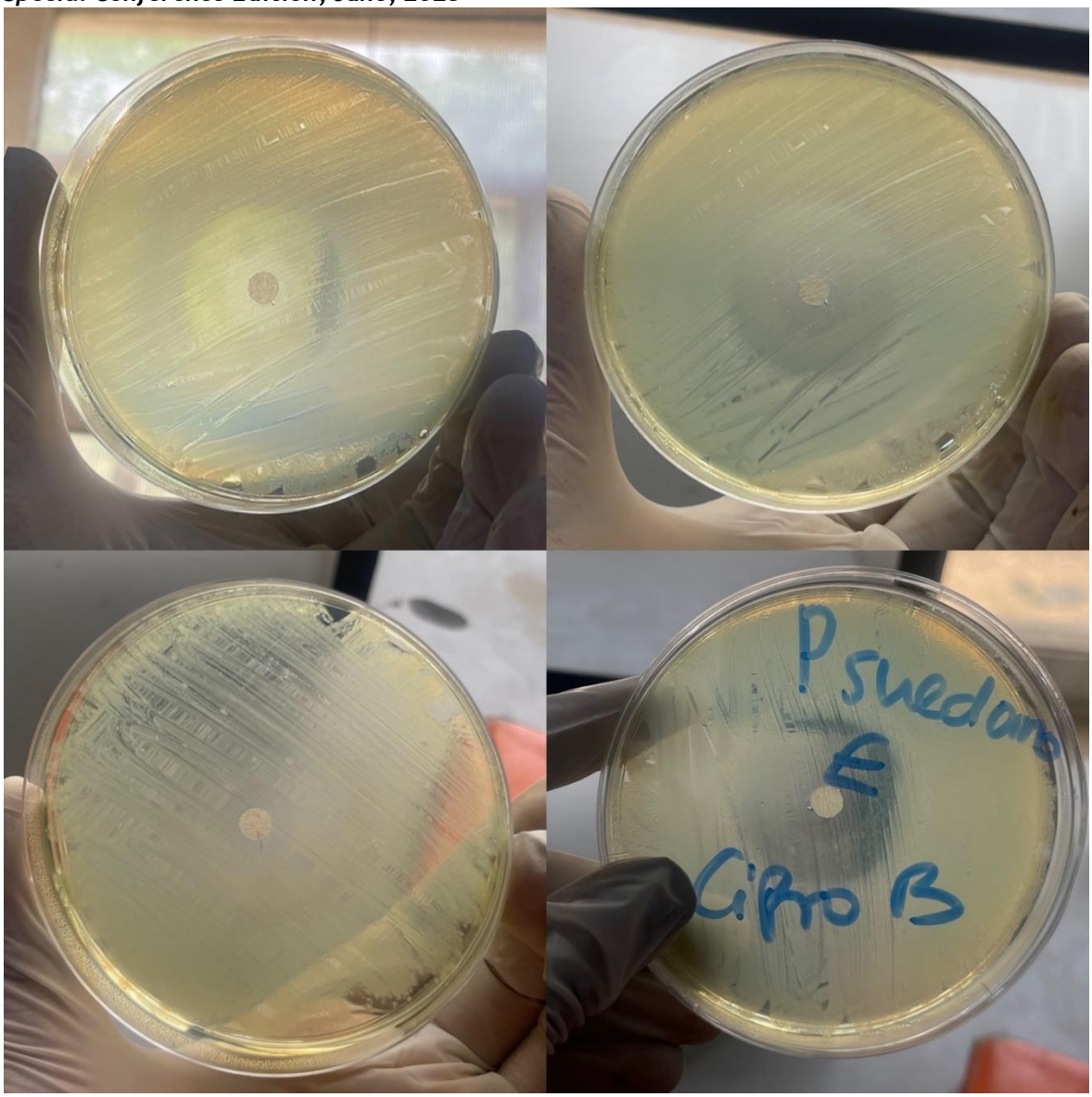

Figure 1: zones of inhibitions of residual antimicrobials against clinical and environmental isolates

The minimum inhibitory concentration of residual antibiotics in urine obtained from P4, P8 and P9 was 25% against *E. coli* bacteria and 50% dilutions in *K. pneumoniae* bacteria. Urine from P1, P2, P5 – P7 were able to inhibit the growth of *K. pneumoniae* and *P. mirabilis* at 25%. Similarly, urine residues from P9 was able to inhibit all the tested environmental isolates at 25% concentration only and samples P4 inhibits the bacteria at 12.5 and 50% concentrations. In addition, samples P5 and P8 were able to inhibit the growth of *E. coli* and *P. aeruginosa* at 25% and 50% concentrations respectively (Table 3).

Table 3: MIC of sampled urine against clinical and environmental bacteria

Clinical isolates	Patient ID/dilution at which MIC occurred									
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
E. coli				0.25				0.25	0.5	
K. pneumoniae	0.25	0.25		0.5	0.25	0.25	0.25	0.5	0.5	
P. mirabilis	0.25	0.25		0.5	0.25	0.25	0.25	0.5	0.5	
Environmental isolates										
E. coli	0.5	0.25		0.25	0.25	0.25	0.25	0.25	0.25	
Shigella spp				0.25	0.5			0.5	0.25	
P. aeruginosa				0.5					0.25	

Note: 0.25 is equivalent to 25%, 0.5 is equivalent to 50%

DISCUSSION

Infectious diseases remain one of the most common causes of morbidity and mortality globally. It is often caused by bacteria, but may also include fungal and viral infections. Report have shown that, about 90% of urinary tract discomfort are caused by bacteria, specifically Gram-negative bacteria while Gram-positive bacteria account for only 10% of cases (Seifu and Gebissa, 2018). With this high rate, the need for use of antimicrobials to manage the situation becomes necessary. In trying to do this, antimicrobials are either overuse or underuse, which can subsequently lead to evolution of resistant bacteria. Due to poor hygiene and environmental pollution in most of our environment, clinically relevant bacteria are now found in the environment, posing risks of transmission to human and animals. Similarly, reports in Nigeria has revealed that the incidence of resistance of bacteria isolated from poultry droppings and cowdung to nitrofurantoin, amoxicillin and sulfamethoxazole-trimethoprim has reached 80 - 90%, while resistance to other beta-lactams and fluoroquinolones ranged from 40-60% (Yusuf and Egbule, 2019).

The fact that all the 10 participants recruited into this study, received antimicrobial treatment, can point to the extent of antimicrobial use (AMU) in the study area. Data on AMU among paediatric inpatients in a tertiary hospital in Nigeria, revealed AMU as high as 80.6% prevalence in 2017, 94.6% in 2018, and 83.6% in 2019. P7 have stayed for 20 days on admission receiving antimicrobial treatment; can suggest possible treatment failure due to resistance. Metronizaole consumption was highest, followed by ceftriaxone (30%). This is in line with the findings of Sekoni *et al*. (2022), who also reported highest consumption of Metronidazole, followed by Ceftriaxone. However, the prevalence of ceftriazone use in this study is low when compared with 66% consumption rate reported by Umeokwonkwo *et al.* (2023) in Abakaliki. The preferred route of administration in this study is oral, against parentherally reported in Umeokwonkwo et al's study. In general, the presence of these antimicrobials in urine samples would have great impact on the isolation and interpretation of urine culture reports, as correct identification of urine antimicrobial activity will help in quantifying the bacterial growth in urine culture. Susceptibility of clinical isolates to the residual antimicrobials in urine samples indicates high concentration of pharmacologically active ingredients in the urine. The insensitivity of *P. aeruginosa* to 9(90%) of the urine samples indicate the resistant nature of the strains obtained from the environmental source. Similarly, the susceptibility of all the isolates tested to the residual antimicrobials in urine sample P9 shows high concentration of active antimicrobial ingredients in the urine. P9 was a female patient admitted into the medical ward with UTI, and has just spent 4 days before the sample was collected. Information on whether the patient has received antibiotic treatments in other hospitals prior to this hospital is not available. It was observed that clinical E. coli is more resistant to the residual antimicrobials in the urine samples than *E. coli* gotten from environmental source. This may be as a result of prior exposure of clinical isolates to antimicrobials, while those in the environments are less exposed. This is worrisome, as the residual antimicrobials can cause a selective pressure by killing susceptible bacteria, and allowing antibiotic-resistant bacteria to develop, especially in this part of the world, where urine are voided freely in the environment and access to antimicrobials is not regulated. The fact that 4-fold dilution of the urine samples can still inhibit the growth of bacteria, also indicate possibility of resistance development, as suboptimal concentration tend to enable bacteria develop resistance faster.

CONCLUSION

The present study demonstrated that commonly used antimicrobials are readily available in concentrations that can kill or inhibit the growth of clinical and environmental bacteria. The result indicates that urine samples of person consuming antimicrobials if not properly disposed, can result in evolution of antibiotic resistance in clinical and environmental bacteria.

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