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ANTIBIOTIC SENSITIVITY PATTERNS AMONGST POST-MORTEM BACTERIAL ISOLATES FROM HIV-INFECTED PATIENTS IN WESTERN KENYA: A CROSS SECTIONAL DESCRIPTIVE STUDY

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Author CMK is the principal author for the paper. He was responsible for acquisition of data, data analysis and drafting the manuscript. Authors IM, PK and DC were responsible for acquisition of data, data validation and reviewing of the manuscript for scientific content. Author AM was responsible for the study design, statistical analyses and interpretation of the results. Authors WE, KKWK and AMS were responsible for overall study design, reviewing the manuscript for scientific content and approval of the final version.

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ABSTRACT

Background: In the sub-Saharan Africa, there are minimal data about the antibiotic sensitivity patterns of the common bacterial isolates from HIV-infected patients. We conducted an autopsy study to determine the antibiotic sensitivity patterns of the common bacterial isolates.
Methods: HIV-infected patients at Moi Teaching and Referral Hospital, Eldoret Kenya, who die while on antiretroviral therapy underwent autopsy. Bacterial cultures on selective media were taken from body fluids and tissues. For instance urine samples were cultured in cysteine lactose electrolyte deficient (CLED) agar; stool in Deoxycholate Citrate Agar (DCA) and Xylose lysine deoxycholate agar (XLD); all other samples, including blood, were cultured in Blood Agar and Mac Conkey. Isolates were stored at -18 to -25°C. They were then sub-cultured and a Gram stain performed to classify the pathogens as either Gram positive or negative. Isolates were

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then subjected to an automated identification and antibiotic sensitivity testing by Minimum Inhibitory Concentration using the Siemens Micro-scan Walkaway 40 plus model® and the Vitek® system.
Results: A total of 416 bacterial isolates were cultured from 202 (57.7%) of the 350 cadavers. The three most common pathogens isolated included *E. coli* (22.4%), *Klebsiella pneumonia* (10.8%) *and Staphylococcus aureus* (9.1%) *E. coli* isolates were highly resistant to ampicillin (97%), 3rd and 4th generation cephalosporins (50%) and gentamicin (40%). 36% of these isolates were Extended Spectrum Beta-Lactamase (ESBL)-producing. *Klebsiella* isolates showed high resistance to ampicillin (86%), ceftazidime (52%) and gentamicin (55%). An estimated 60% of *S. aureus* isolates were methicillin resistant *Staphylococcus aureus* (MRSA) and 13% were vancomycin resistant *Staphylococcus aureus* (VRSA). *Enterococcus spp* showed high level resistance to gentamicin- and streptomycin-high level synergy (>80%). An estimated 5% of the isolates were vancomycin resistant *Enterococci* (VRE).
Conclusion and Recommendation: Our data show that antibiotic-resistant bacterial pathogens colonize HIV-infected patients at the time of death. This study provides definitive evidence that MRSA, VRE and ESBL Gram-Negative-Rods are an emerging issue in our HIV-infected population.

Keywords: Antibiotic sensitivity; HIV-infected; autopsy.

1. INTRODUCTION

Antibiotic resistance is a cause of increasing morbidity and mortality globally. It is responsible for prolonged inpatient hospitalization, treatment failure and escalating healthcare costs [1-3]. For instance, according to the US Centers for Disease Control and Prevention (CDC) an estimated 99,000 deaths occur annually in the US as a result of hospital acquired infections, making them the leading cause of infectious death and one of the top 10 causes of death overall [4]. Methicillin resistant S*taphylococcus aureus* (MRSA), vancomycin resistant *Enteroccocii* (VRE) and *Clostridium difficile* account for at least 350,000 of these infections and are responsible for approximately 12,000 deaths annually [4]. In the European Union, an estimated 25,000 patients die annually of a serious resistant bacterial infection acquired in hospitals [5]. Though resistance rates are well characterized in many resource replete settings, such data are limited in resource constrained settings such as the sub- Saharan Africa. A recent systematic review including studies on antibiotic susceptibility from Sub-Saharan Africa and Asia found that many commonly used antibiotics face considerable resistance in the most common bacterial pathogens [6].

In Kenya, the extent and impact of antibiotic resistance is not well documented. However, limited data available indicate that Kenya is already experiencing high levels of antibiotic resistance, which is rapidly rising [7]. For instance high rates of resistance have been found for respiratory infections, enteric infections and for infections acquired in healthcare facilities [8-16]. This suggests that many antibiotic regimens supplied by the government are unlikely to be effective against infections of wide concern [7].

HIV infected individuals are at increased risk of developing infections, including infections caused by resistant bacterial pathogens. In order to quantify the burden of antibiotic resistance in this population, we conducted antibiotic sensitivity tests on bacterial isolates from autopsy samples of HIV infected patients at the Moi Teaching and Referral Hospital in western Kenya.

2. METHODS

2.1 Ethical Approval and STUDY Design

The study was approved by the Institutional Research and Ethics Committee of the Moi University and Moi Teaching and Referral Hospital. Prior to recruitment into the study, written informed consent was obtained from the next of kin of the deceased subjects. We conducted a prospective cross-sectional descriptive study between July 2009 and June 2013.

2.2 Setting

The study was conducted at the Moi Teaching and Referral Hospital (MTRH), in Eldoret Kenya. This is the largest regional referral centre in western Kenya and serves as the teaching hospital for Moi University School of Medicine. It serves a catchment population of 16 million people (40% of Kenya's population). The total bed capacity at MTRH is 720 with 140 beds in two adult general medical wards. Due to resource limitation, it is common for inpatients to share beds. There is no isolation facility for patients with suspected infectious diseases. Between 40 and 50% of all adult general medical ward bed occupancy is by HIV-infected patients.

MTRH, in partnership with Moi University School of Medicine, Ministry of Health and a consortium of North American Universities led by Indiana

University has established an ambulatory HIV treatment program called Academic Model Providing Access to Healthcare (AMPATH). AMPATH has established a robust ambulatory HIV care program with over 150,000 HIV-infected patients enrolled so far. AMPATH provides comprehensive HIV care including free anti-retroviral therapy, anti-tuberculous medications, isoniazid- and cotrimoxazole- preventive therapy, therapeutic food supplements, multivitamins and hematinics. The adult ART treatment guidelines in use during the period of this study recommended initiation of treatment for patients with a CD4 count of ≤350 cells/ul or with WHO clinical stage 3 or 4 disease. Typically, AMPATH patients who need inpatient treatment are hospitalized at MTRH.

2.3 Population

Study subjects were AMPATH enrolled, deceased HIV infected individuals, aged ≥ 13 years who had died within 12 months of initiating ART. Subjects were recruited whether the death had occurred in hospital or at home. A research assistant checked the details of all the bodies arriving at the MTRH morgue on a daily basis. Using these details, AMPATH enrolled patients were identified from an electronic medical database. For subjects meeting the inclusion criteria, the next of kin were contacted and asked for consent for autopsy. Subjects were enrolled into the study after obtaining informed consent from the next of kin.

2.4 Procedure for Autopsy and Specimen Collection

Cadavers of eligible subjects were stored at 2 to 5°C pending autopsy examination. Autopsies and specimen collections were performed within 48 hours of death.

Specimens were collected aseptically from each cadaver in a stepwise manner. First, 10 mls of Cerebrospinal fluid (CSF) was tapped from the inter-vertebral space of all cadavers using a gauge-18 spinal tap needle in an aseptic technique. Next, 30 ml of blood was aspirated from the heart using a gauge-12 long needle in an aseptic fashion. A sterile hot spatula was used to open the heart for collection of blood if the blood had clotted and could not be aspirated. The blood was then injected into blood culture bottles (10 ml in aerobic and 10 ml in anaerobic media) using aseptic technique.

Bone marrow sample was aspirated from the iliac crest or sternum using a bone marrow aspirate needle in an aseptic technique. The aspirated bone marrow was then injected into culture bottles (aerobic and anaerobic). A sample of urine was aspirated aseptically from the bladder (trans-abdominally) and put into culture bottles.

Tissue samples from the brain and meninges, lung and pleura, lymph nodes, pericardium and heart were collected from each cadaver. Other tissues collected included the stomach, ileum, colon, liver, spleen, adrenal, kidney, bladder, cervix and uterus, testes, ovary, muscle and skin (lesions).Any abnormal collections of fluid such as ascites, pleural effusion and pus were also collected. These samples were delivered to the microbiology lab immediately for processing.

2.5 Lab Processing

Collected samples were then cultured in selective growth media. For instance urine samples were cultured in cysteine lactose electrolyte deficient (CLED) agar; stool in Deoxycholate Citrate Agar (DCA) and Xylose lysine deoxycholate agar (XLD); all other samples, including blood, were cultured in Blood Agar and Mac Conkey.

Isolates were stored at -18 to -25°C. They were then sub-cultured and a Gram stain performed to classify the pathogens as either Gram positive or negative. Isolates were then subjected to an automated identification and antibiotic sensitivity testing by Minimum Inhibitory Concentration using the Siemens Micro-scan Walkaway 40 plus model® and later Vitek® system.

Lab processing was done at the AMPATH special microbiology lab and at the KEMRI/Walter Reed reference lab in Kericho. All microbiological analyses were conducted as per the standards set by Clinical and Laboratory Standards Institute (CLSI) [17]. Lab print-outs contained information on the identity of the organism and its antibiotic sensitivity pattern based on preselected antibiotic panels for Gram positive and negative organisms as per CLSI guidelines.

2.6 Data Cleaning and Storage

Data from the lab print-outs were directly keyed into a customized computerised database WHONET 5.6 [18]. This software was developed by the World Health Organization for the storage and analysis of drug resistance data. Data were stored on password protected hard-drives with access limited to authorized personnel only.

2.7 Statistical Analysis

Analysis involved mainly descriptive statistics of the sample. Frequencies and proportions of the common isolates were computed and tabulated. For each pathogen species with ≥10 isolates, the proportion resistant to the commonly used antibiotics, together with their 95% CI were computed. Any pathogen with less than ten isolates was excluded from this analysis of resistance patterns to reduce the effect of random error. Organisms of the same genus which had similar resistance patterns were analyzed together. Among the *Enterobacteriacea*, the proportions of ESBL-producing isolates were also computed.

3. RESULTS

350 cadavers were included in this study. Of these, 202 (57.7%) had positive bacterial cultures. Overall, 416 bacterial isolates were cultured since some of the cadavers had more than one isolate recovered. The three most common pathogens isolated included *E. coli* (22.4%), *Klebsiella pneumonia* (10.8%) *and Staphylococcus aureus* (9.1%). Table 1 shows the frequencies and proportions of the ten most common pathogens isolated. Pathogens with less than ten isolates were grouped together under 'others'. Other medically important pathogens isolated included *Proteus mirabilis (1.2) and Pseudomonas aeruginosa* (0.7%).

We considered pathogens isolated from potentially sterile sites. As illustrated in Table 2, over 60% of the isolates were recovered from blood, lung and spleen. The most common isolates from these potentially sterile sites included *E. coli, K. pneumoniae* and *S. aureus*. Tables 3.1- 3.5 show the antibiotic resistance patterns of the 5 most common pathogens beginning with gram positive organisms, Tables 3.1-3.3 and then gram negative organisms, Tables 3.4-3.5.

Among the gram positive organisms, *staphylococcus spp* was the most common. Table 3.1 illustrates the resistance pattern of *Staphylococcus aureus isolates*. An estimated 60% of *S. aureus* isolates were *MRSA* based on their resistance to oxacillin which was used as a surrogate for methicillin in this study. More than 40% of the isolates were resistant to both gentamicin and clindamycin. An estimated 13% of *S. aureus* isolates were resistant to vancomycin.

Other important gram positive pathogens isolated included the *Enterococcus spp*. The resistance profiles of *E. faecalis* and *E. faecium* are presented in Tables 3.2 and 3.3 respectively. As illustrated, the *Enterococcus spp* showed high level resistance to gentamicin- and streptomycin-high level synergy, with over 80% of isolates being resistant. However, only an estimated 4% of *E. faecalis* isolates and 7% of *E. faecium isolates* were resistant to vancomycin.

Among the Gram negative pathogens, *E. coli* was the most common. The antibiotic resistance profile of *E. coli* is shown in Table 3.4. As illustrated, nearly 100% of the isolates were resistant to ampicillin; an estimated 50% were resistant to both 3rd and 4th generation cephalosporins and nearly 40% were resistant to gentamicin. However, the isolates showed minimal resistance to nitrofurantoin (7.5%), amikacin and meropenem (1.1% each). Over 36% of these isolates were ESBL-producing.

The antibiotic resistance profile of the *Klebsiella spp* which included *K. Pneumoniae and K. Oxycota* isolates is shown in Table 3.5. As illustrated, majority of the isolates were resistant to ampicillin (86%), ceftazidime (52%) and gentamicin (55%). A smaller proportion of the isolates were resistant to ciprofloxacin and amikacin, with meropenem showing the least resistance (3%). An estimated 10% of the *Klebsiella spp* isolates were ESBL producing.

Table 1. Overall frequency and proportions of the most common isolates

Organism	Frequency (n=416)	Proportion (%)
E. coli	93	22.4
Klebsiella pneumonia	45	10.8
Staphylococcus aureus	38	9.1
Staphylococcus sciuri	28	6.7
Enterococcus faecalis	25	6.0
Citrobnacter freundii	17	4.1
Enterococcus faecium	16	3.8
Staphylococcus hemolyticus	16	3.8
Enterobacter cloacae	15	3.6
Klebsiella oxycota	14	3.4
Salmonella spp	13	3.1
Morganella morganii	12	2.9
Others	84	20.2

Table 2. Frequency of pathogens isolated from sterile sites

Specimen type	Number and proportion of isolates (n=268)			Top five isolates		
	No.	Proportion (%)	Number of cadavers	Pathogen	No.	Proportion (%)
Blood	94	35.0	85	*E. coli*	15	16
				K. pneumoniae	10	10.6
				S. aureus	9	9.6
				C. freundii	7	7.4
				E. faecium	5	5.3
Spleen	41	15.3	38	*E. coli*	12	29.3
				K. pneumoniae	4	9.8
				S. aureus	3	7.3
				E. faecium	3	7.3
				Salmonella ssp	2	4.9
Cerebrospinal fluid	38	14.2	32	*E. coli*	7	18.4
				Staphylococcus spp	6	15.9
				Klebsiella spp	4	11
				E. faecalis	4	11
				Salmonella spp	2	5.3
Bone marrow	36	13.4	35	*E. coli*	7	19.4
				Staphylococcus spp	6	16.6
				Salmonella spp	3	8.3
				E. faecalis	3	8.3
				K. pneumoniae	2	5.6
Urine	24	9.0	23	*Staphylococcus spp*	6	24
				K. pneumoniae	4	16.7
				E. coli	2	8.3
				E. faecalis	2	8.3
				E. faecium	2	8.3
Others (Lymph node, adrenal gland, ascitic fluid)	35	13.1				
Total	268	100				

Table 3.1. Resistance pattern of *Staphylococcus aureus*

Antibiotic	No. of isolates	Proportion (%) resistant (95% CI)
Oxacillin*	38	60.5 (43.4-75.5)
Gentamicin	38	42.1 (26.7-59.1)
Clindamycin	38	55.3 (38.5-71.0)
Vancomycin**	38	13.2 (5.0-28.9)
Co-trimoxazole	37	75.7 (58.5-87.7)

**Oxacillin was used as a surrogate for methicillin. 60% of S. aureus were MRSA, ** 13.2% of S. aureus were vancomycin resistant (VRSA)*

Table 3.2. Resistance pattern of *Enterococcus faecalis*

Antibiotic	No. of isolates	Proportion (%) resistant (95% CI)
Ampicillin	18	11.1 (1.9-36.1)
Gentamicin (high-level synergy)	19	89.5 (65.5-98.2)
Streptomycin (high-level synergy)	18	83.3 (57.7-95.6)
Vancomycin	23	4.3 (0.2-23.9)
Linezolid	23	4.3 (0.2-23.9)

Table 3.3. Resistance pattern of *Enterococcus faecium*

Antibiotic	No. of organisms	% resistant (95%CI)
Ampicillin	12	100 (69.9-100)
Gentamicin (high-level synergy)	11	81.8 (47.7-96.8)
Streptomycin (high-level synergy)	12	91.7 (59.8-99.6)
Vancomycin	15	6.7 (0.4-34.0)
Linezolid	16	6.2 (0.3-32.2)

Table 3.4. Resistance pattern of *E. coli*

Antibiotic	No. of isolates	Proportion (%) resistant (95% CI)
Ampicillin	93	97.8 (91.6-99.6)
Ceftriaxone	85	52.9 (41.8-63.7)
Cefipime	93	49.5 (39.0-60.0)
Ciprofloxacin	93	65.6 (55.0-74.9)
Gentamicin	93	37.6 (27.9-48.3)
Amikacin	93	1.1 (0.1-6.7)
Meropenem	93	1.1 (0.1-6.7)
Nitrofurantoin	93	7.5 (3.3-15.4)
Co-trimoxazole	93	95.7 (88.7-98.6)
ESBL-producing	34	Proportion ESBL-producing 36.6%

Table 3.5. Resistance pattern of *Klebsiella* species (*K. pneumoniae, K. oxycota*)

Antibiotic	No. of isolates	Proportion (%) resistant (95% CI)
Ampicillin	59	86.4 (74.4-93.5)
Ceftazidime	59	52.5 (39.2-65.5)
Cefipime	59	25.4 (15.4-38.7)
Ciprofloxacin	59	22 (12.7-35.0)
Gentamicin	59	55.9 (42.4-68.6)
Amikacin	59	11.9 (5.3-23.6)
Meropenem	59	3.4 (0.6-12.8)
Nitrofurantoin	59	32.2 (21.0-45.80)
Co-trimoxazole	59	78 (65.0-87.3)
ESBL-producing	6	Proportion ESBL-producing 10.2%. All were *K. pneumoniae* isolates

Citrobacter freundii isolates showed high resistance to ampicillin (93%); ceftriaxone and gentamicin (>50%) but minimal resistance to amikacin and meropenem (11 and 6% respectively). An estimated 17% of these isolates were ESBL producing.

Enterobacter cloacae isolates showed high resistance to ampicillin; 3rd and 4th generation cephalosporins; ciprofloxacin and gentamicin, with 70-100% of the isolates being resistant. However, only a small proportion of the isolates showed resistance to amikacin and meropenem (13 and 7% respectively). An estimated 33% of the isolates were ESBL producing.

Among *Salmonella spp*, all the isolates were resistant to ampicillin (100%), and 70% were resistant to 3rd and 4th generation cephalosporins. However, *Salmonella* isolates showed lower resistance to ciprofloxacin (23%) and minimal resistance to levofloxacin, amikacin and meropenem (approximating 0%). Nearly 40% of the isolates were ESBL producing.

Nearly 100% of *Morganella morganii* isolates were resistant to ampicillin. However, less than 10% of the isolates were resistant to 3rd and 4th generation cephalosporins, amikacin and meropenem. An estimated 8% were ESBL producing

4. DISCUSSION

Our data show a high prevalence of bacterial isolates recovered from autopsy samples from this HIV-infected population. Nearly 60% of the cadavers had positive bacterial cultures. It should however be appreciated that these isolates are not necessarily of clinical significance but may be mere colonizers. The

antibiotic susceptibility patterns also demonstrate a high prevalence of antibiotic resistant pathogens in this setting. Although the antibiotic resistance profiles may not be clinically relevant, they are important from an epidemiological standpoint. It is conceivable that colonization by resistant bacterial pathogens could result in infection when the opportunity arises, especially among the immune-compromised population.

Over 60% of the isolates were recovered from potentially sterile sites, including blood, spleen and cerebral spinal fluid. Among these, the most common isolates included *E. coli, K. pnuemoniae* and *S. aureus*. Although meticulous care was taken to collect the samples aseptically, it is likely that *E. coli* and *S. aureus* were contaminants and that *K. pnuemoniae* is the leading pathogen in this population. However many ante-mortem studies in Africa have frequently implicated *E. coli* and *S. aureus* among the common causes of bacteremia, and their role in causing invasive disease is not to be downplayed [15,19-25].

Among the gram positive pathogens, *Staphylococcus aureus* showed high resistance to most of the commonly used antibiotics. Over 50 % of the isolates were resistant to clindamycin and co-trimoxazole. An estimated 60% of the isolates were MRSA. Only the glycopeptide vancomycin retained significant activity against these isolates. This resistance profile for *Staphylococcus aureus*, to a large extent, limits treatment options for infections caused by this pathogen in our setting where vancomycin is not routinely available. Other studies in Africa and across the globe have documented MRSA prevalence ranging between 15% and 55% [26-31]. Our study documented higher prevalence of MRSA probably due to inclusion of repetitive isolates of the same pathogen from different samples of a given cadaver.

The *Enterococcus spp* showed high level resistance to aminoglycoside-high level synergy, with over 80 % of the isolates being resistant. Nearly 100% of *Enterococcus faecium* isolates were resistant to ampicillin. Only vancomycin and linezolid appear to be effective against the *Enterococcus spp* in our setting. However, an estimated 5% of the isolates were resistant to vancomycin and linezolid. VRE is of increasing importance in causing multi-drug-resistant healthcare acquired infections across the globe. Data on the epidemiology of VRE in Africa is limited. A study in Algeria documented a prevalence of 3.2% among clinical cases attending a University Hospital [32]. In Egypt, a 9.5% VRE carriage rate was reported among Intensive Care Unit (ICU) personnel [33]. In South Africa, over 80% of *Enterococci* recovered from groundwater were resistant to vancomycin [34]. Data from Europe and the US indicate varying prevalence ranging between <2% in Finland to 33% in the US [35]. The higher prevalence of VRE in the developed world is attributed to higher usage of vancomycin for MRSA especially in intensive care units; higher community colonization by VRE due to usage of a vancomycin-like glycopeptide, avoparcin, in animal husbandry and better diagnostic facilities [35]. Co-endemicity of VRE and MRSA can facilitate the horizontal transfer of *vanA*- or *vanB*-containing transposons, which transforms MRSA into *Vancomycin Resistant Staphylococcus aureus* (VRSA) [36]. This has serious implications for patient care especially in a resource constrained setting such as ours in which we found VRSA prevalence of 13%.

Among the *Enterobacteriacea*, *E. coli* isolates showed high resistance to ampicillin and 3rd and 4th generation cephalosporins. However, only a small proportion of the isolates were resistant to nitrofurantoin, amikacin and meropenem. Thus, in our setting nitrofurantoin can still be used to treat urinary tract infections caused by *E. coli* whereas amikacin can be reserved for more invasive disease. However, nearly 40% of *E. coli* and *Salmonella spp* isolates and 10% of *K. pneumoniae isolates* were ESBL-producing. The prevalence of ESBL-producing *Enterobacteriacea* in other African settings is highly variable and ranges between 15-75% in various studies [25,37-40]. ESBLs are enzymes capable of hydrolyzing penicillins, broad-spectrum cephalosporins and monobactams. Many ESBL-producing organisms are multidrug resistant and are commonly resistant to aminoglycosides and floroquinolones as well. Only carbapenems are considered effective for the treatment of such infections. Thus a high prevalence of ESBL-producing *Enterobacteriacea* in our setting where carbapenems are not routinely available is worrisome.

This study highlights the silent epidemic of antibiotic resistance in a setting where limited data are available. It should serve to raise awareness of this important global challenge and encourage health care professionals to develop appropriate measures to curb the spread of resistant pathogens.

4.1 Strengths and Limitations

A major strength for this study is that we utilized state-of-the-art automated AST technology (Microscan ®) which uses the minimum inhibitory concentration method to classify agents as being resistant or susceptible. This is more reliable than the traditional manual methods.

One limitation for this study is that we could not determine the clinical significance of the bacterial

pathogens isolated. It is possible that some of them were only colonizing the host and were not responsible for disease. However, the data is still important epidemiologically since it depicts the prevalence of antibiotic resistant pathogens in our environment, which ultimately have the potential to cause disease. Another limitation is that we included all the isolates recovered in the analysis without considering whether or not they were repetitive isolates.

5. CONCLUSION

Our data show that antibiotic resistant bacterial pathogens colonize HIV-infected patients at the time of death. It is evident that MRSA, VRE, ESBL Gram Negative Rods (GNRs) including aminoglycoside resistant GNRs are an emerging issue at least in our HIV-infected population.

6. RECOMMENDATION

In Kenya there has been little concern for antibiotic resistant bacteria when choosing antibiotic regimens and when stocking hospitals by the Ministry of Health. Our data suggests that monitoring antibiotic sensitivity patterns in our hospitals should be a key element in deciding what antibiotics to stock. Future research efforts should be directed at clinical isolates in order to develop local antibiograms and to formulate antibiotic stewardship programs.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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