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Patterns of Multidrug Bacterial Clinical Isolates and Cytokine Responses to Antibiotic Misuse in Nnewi, Anambra State, Nigeria

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ABSTRACT: Multidrug-resistant (MDR) bacterial pathogens can pose significant health-care challenges by rendering most antimicrobials ineffective. Hence the objective of this paper was to evaluate the patterns of multidrug bacterial clinical isolates and cytokine responses to antibiotic misuse in Nnewi, Anambra State, Nigeria using appropriate standard methods. Also, the levels of tumor necrosis factor alpha (pg/ml), interferon-gamma (pg/ml), and interleukin-10 (pg/ml) in post-surgical patients and control subjects were evaluated using enzyme link immunosorbent assay method.The prevalence of wound infection among study participants was (20%). *Pseudomonas aeruginosa* (40%) and *Escherichia coli* (19%) were most likely to be associated with wound infection. The MDR bacterial isolates shows highest resistance to Cefixime (80%) and Ciprofloxacin (80%) in the surgical wound infection patients. The levels of tumor necrosis factor alpha, interferon-gamma and interleukin-10were significantly higher in thepost-surgical wound infection patient as compared with the control group ($p<0.002$). MDR-bacterial infections are more virulent, and the observed excessive inflammatory response may impede infection resolution, which may help to explain in part, the poor treatment outcome in patients with MDR infections, even after prompt antibiotic treatment.

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Antibiotic resistance in bacterial isolates has spread widely as a result of the regular use of antimicrobial agents in both human and veterinary medicine, particularly in Gram negative bacteria, with antibiotic selective pressure, these resistant bacteria species tend to persist, allowing the organism to cause additional infections such as septicaemia (Catalano *et al*., 2022). Antimicrobials and other antibacterial medications

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should only be administered with a prescription. However, a combination of factors, including a shortage of licensed prescribers and the proliferation of unregulated patent medicine vendors, has resulted in a crisis of irrational drug use, exacerbating antimicrobial resistance globally including Nigeria (Catalano *et al*., 2022). Multidrug-resistant (MDR) bacterial pathogens can pose significant health-care challenges by rendering most antimicrobials ineffective (Catalano *et al*., 2022, Makanjuola *et al*., 2018, Egbule *et al*., 2016). MDR pathogens can develop resistance to various antimicrobials through horizontal gene transfer and genetic mutations caused by drug overexposure (Makanjuola *et al*., 2018).Antibiotic resistance causes more than 60,000 deaths in Nigeria each year (Kany *et al*., 2019). Already alarming, the global death toll is expected to rise to more than 10 million by 2050 (Walsh *et al*., 2023). Surgical wound infections develop due to three factors: (i) the degree of bacterial contamination during the operation, (ii) the duration of the procedure, and (iii) the patient's underlying disease, such as immune deficiency, diabetes, and malnutrition (5). Multidrug-resistant bacteria isolates have presented significant challenges to the treatment of surgical wound infections worldwide(Nobel *et al*., 2022). Exposing retinal and microglial cells to MDR bacterial strains results in increased levels of IL-6, IL-1α, IL-8, IL-10, tumor necrosis factor-alpha, and interferon-gamma (Naik *et al*., 2021). Thus, MDR-bacterial infections are more virulent, and an excessive inflammatory response may impede infection resolution. Multidrug resistance in bacteria is frequently caused by the accumulation of resistance plasmid genes as a result of antibiotic abuse; additionally, once incorporated into an integrin, the resistance gene becomes tagged, allowing it to easily transfer to closely related bacteria(Davies and Davies, 2010). Another mechanism of multidrug resistance is the active pumping of drugs by multidrug efflux pumps, which are typically chromosomally mediated(Nishino *et al*., 2021). Indeed, there is a strong correlation between antibiotic use and the frequency of MDR bacterialin Nigeria(Makanjuola *et al*., 2018). There is also an ongoing need to define the interplay of immune responses to unnecessary/misused antibiotics in Nigeria, taking into account that many interdependent players, such as inflammatory cells and cytokines, participate to varying degrees in preventing antibiotic misuse. As a result, the objective of this paper was to evaluate the patterns of multidrug bacterial clinical isolates and cytokine responses to antibiotic misuse in Nnewi, Anambra State, Nigeria.

MATERIALS AND METHODS

*Sample collection and biochemical analysis:*The study included 100 hospitalized patients with post-operative surgical wounds from the general surgery, obstetrics/gynecology, and orthopedic wards of Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi, Anambra state, Nigeria and one hundred apparently healthy individual, ranging in age from 17 to 80 years. Prior to collecting samples, an informed consent form was signed. Samples were only collected from patients prior to surgical wound dressing. Each patient with post-surgical wound provided two swab samples collected with sterile swab sticks and placed in an Amies transport medium.All collections were conducted in strict aseptic conditions. Specimens were transported to the laboratory for analysis within 3-4 hours of collection. Each subject provided five (5ml) venous blood samples from the medial cubital vein using a vacutainer and needle, which were then placed in plain containers.The serum collected after an overnight clot and was used to determine the levels of tumor necrosis factor alpha (pg/ml), interferon-gamma (pg/ml), and interleukin-10 (pg/ml) in post-surgical patients and control subjects using enzyme link immunosorbent assay method.Structured questionnaires were used to collect information including demographic data, existing chronic disease (such as diabetes mellitus), past medical history, current drug use, smoking, length of preoperative hospital stay, and antimicrobial prophylaxis.

*Bacteriological analysis:*The wound swab specimens were inoculated onto Blood agar, MacConkey agar, and Mannitol salt agar plates and incubated aerobically at 37^0C for 24 hours. Duplicate blood agar plates were incubated anaerobically at 37^0C for 24 hours. Bacterial isolates were identified morphologically and biochemically. All of the media used (blood agar, MacConkey agar, and Mannitol salt agar) were prepared in accordance with the manufacturer's instructions.

Isolation and identification: Macroscopy: Isolates were identified by their colonial appearance on agar. *P.aeruginosa* produces pale colonies on MacConkey agar, whereas *E.coli* produces pink colonies. On nutrient agar, *P.aeruginosa* produces greenish colonies, while *E.coli* appears colorless and *S.aureus* produces golden yellow colonies on mannitol salt agar. Morphological characteristics such as size, form, elevation, opacity, odor, and edge were used to identify the organisms. Prior to this, Gram's stained techniques were used to create films from the swabs and colonies, which served as a guide to determining whether the organisms were Gram positive or Gram negative.Following the initial identification of the

organism using Gram staining techniques, the colonies on MacConkey agar were divided into lactose and non-lactose fermenting colonies. The non-lactose fermenting and lactose fermenting colonies were subjected to the necessary conventional biochemical tests such as citrate utilization, urea production, indole production, oxidase test, motility test, and sugar fermentation (maltose, sucrose, and mannitol) test.

*Bacterial cultivation and pure culture preparation:*All isolates were placed on MacConkey agar plates and incubated at 37ºC for 24 hours. The cultured plates were examined for growth and appearance. A subculture was created from each isolate's colonies. This was accomplished by removing portions of the colonies with a sterile wire loop and streaking them on freshly prepared nutrient agar plates. The plates were incubated at 37°C for 24 hours. All isolates were identified phenotypically and biochemically using standard protocols.

*Antibiotics Susceptibility Testing:*Antimicrobial susceptibility testing was carried out on each isolates by the disc diffusion method using the Kirby- Bauer disc diffusion method in accordance with the National Committee for Clinical Laboratory Standards guideline to evaluate the sensitivity of the test organisms to the various antibiotics. Test isolates were grown on Mueller-Hinton agar and incubated at 37°C for 24 hours. Colonies were suspended in sterile normal saline, and the inoculum density was adjusted to meet 0.5 McFarland turbidity standards.A sterile cotton wool swab was inserted into each test tube containing the standardized inoculum suspension, rotated with firm pressure on the inside wall to remove excess fluid, and then swabbed the surface of a freshly prepared dried Mueller-Hinton agar plate. The antimicrobial disc used includes Ceftazidime (Caz 30µg), Gentamycin (GN 30µg), Ofloxacin (ofl 5µg), Ciprofloxacin (Cpr 5µg), Erythromycin (Ery 10ug), Imipenem (lmp 10ug), Oxacillin (oxa, 1ug), Cefuroxime (Crx 30ug), Cefixime (CXM 5ug), and Augmentin (Aug, 30ug) (Oxide).The disc was placed on the surface of the inoculated Muller Hinton agar plate and incubated at 370C for 24 hours. After incubation, diameters of zones of inhibition were measured to the nearest millimeter using a transparent meter rule. The clinical isolates diameter zones were compared with reference control organism *Escherichia coli* ATCC 25922 and were interpreted as susceptible or resistant according to the CLSI (2000).

Statistical Analysis: All data were analysed using Statistical Package for Social Sciences (SPSS) for windows, version 21. Non parametric date such prevalence of post-surgical wound infections were expressed as percentage. Student (t) test was used for the comparison of parametric data of individuals with post-surgical infections and controls. The changes were considered significant, when p-values were less than 0.05.

RESULTS AND DISCUSSION

A total of one hundred (100) post-operative wound swabs specimens from hospitalized patients at NAUTH and one hundred apparently healthy individual were analyzed. The prevalence of wound infection among study participants was (20%) (Table 1). *Pseudomonas aeruginosa* (40%) and *Escherichia coli* (19%) were most likely to be associated with wound infection (Table 2). The MDR bacterial isolates shows highest resistance to Cefixime (80%) and Ciprofloxacin (80%) in the surgical wound infection patients (Table 3). Table 5 shows the mean ± (SD) Levels of tumor necrosis factor alpha, interferon-gamma and interleukin-10in the post-surgical wound patient as compared with the control group. The levels of tumor necrosis factor alpha, interferon-gamma and interleukin-10were significantly higher in the post-surgical wound infection patient as compared with the control group ($p<0.002$).

Table 1: Prevalence of surgical wound infection

Variable	N	No. infected (%)
NAUTH	100	20(20)

N =Number of sample examined

Table 2: Antibiotic susceptibility pattern of clinical isolates

Isolates	N	R (%)	S (%)
P.aeruginosa	19	15 (79)	4(21)
E.coli	20	15 (75)	5 (25)
Proteus mirabilis	4	3 (75)	1 (25)
Staph aureus	7	5 (71.4)	2 (28.6)
Total	50	38 (76)	12(24)

Key: N=Number of clinical isolates; R=Resistant, S=Sensitive

A total of one hundred (100) post-operative wound swabs specimens from hospitalized patients at NAUTH and one hundred apparently healthy individuals were analyzed. The prevalence of wound infection among study participants was (20%) (Table 1).Although this is lower than an earlier report of 70.1% (Oladeinde *et al.*, 2013), however, this could be as a result of improved sanitary condition of this study site. *Pseudomonas aeruginosa* (79%) was found to be the most common etiologic agent of surgical wound infections in NAUTH, followed by *Escherichia coli* (75%), *P. mirabilis* (75%), and *E. coli* (71.4%) (Table 2). However, it has been reported that *P. aeruginosa* is most commonly recovered from post-operative surgical wounds, possibly due to its high survival rates in the hospital setting (Wood *et al.*, 2023). Also, *Pseudomonas aeruginosa* has been reported to rank second among nosocomial pathogens isolated from hospitals, often contaminating hospital equipments

such as wound dressing sinks and other surgical apparatus, and even antibiotic-resistant strains can survive in supposedly sterile equipments used in hospitals, making it a dangerous nosocomial pathogen widely distributed in hospital environments where they are particularly difficult to eradicate (Wood *et al.*,2023).The antibiotic susceptibility results revealed that the bacteria isolates were highly resistant to Cefuroxime (80%), followed by Cefixime (75%), Augmentin (60%), Ofloxacin (54%), Gentamycin (50%), and Erythromycin. The isolates' high resistance to the various antimicrobial agents used in this study could be attributed to a number of factors, including inappropriate antibiotic use and antibiotic pressure-induced drug inhibition mechanisms. It has also been reported that the presence of bacterial drug-inhibiting enzymes such as cephalosporinase and penicillinase aminoglycoside acetyltransferase reduces the effectiveness of commonly used antibiotics (Kakoulli*et al.,* 2021).

Table 3: Antibiogram of bacteria isolates

Class of Antibiotics	Type of antibiotics	NAUTH N (%) N=50	
		R	S
Penicillin	Augmentin (30µg)	30(60)	15(30)
	Oxacillin (1µg)	0()	5(10)
Aminoglycoside	Erythromycin (10µg)	25(50)	25(50)
	Gentamycin (30µg)	25(50)	25(50)
Cephalosporin	Ceftazidime (30µg)	20(40)	30(60)
	Cefuroxime (30µg)	40(80)	10(20)
	Cefixime (5µg)	35(70)	15(30)

Key: N=Number of isolates; R=Resistant, S=Sensitive

Table 4: Antibiotics misuse awareness report amongst studied population

Awareness of antibiotics misuse	Respondents (%)
Knowledgeable	10 (10)
Average Knowledge	20 (20)
Poor Knowledge	70 (70)
Total	100

Table 5: Levels (mean ± SD) of Tumor necrosis factor alpha (pg/ml), Interferon-gamma (pg/ml) and Interleukin-10 (pg/ml) in post-surgical patients and control subjects

Time intervals	TNF-alpha	Interferon-gamma	Interleukin-10
P	348 ± 0.05	128 ± 0.06	23.24 ± 0.40
C	23.87 ± 0.22	15.68 ± 3.43	1.23 ± 1.76
P value	0.001*	0.001*	0.001*

ns = non-significant * = significant; P = Post-surgical wound infection patients; C = Control subjects

Evidently, the study shows a prevalence (70%) (Table 4) in the number of respondents with a poor knowledge of an awareness that antibiotics can be misused. This is when compared with those who are knowledgeable and averagely knowledgeable. The data is worrisome and would necessitate utilizing various mass media for sensitization and enlightenment in espousing the dangers in self-medication and advocating for an immediate stop.Aside from the human cost, there is also an enormous economic cost to ignoring MDR bacterial strains. As drug resistance spreads, healthcare costs will rise, and sustainable food sources will face increased strain. According to these projections, the World Bank Group estimates that up to 24 million people worldwide will be forced into extreme poverty by 2030.The primary burden of this economic downturn will fall on already struggling low-income countries, and the global impact could be comparable to the 2008 financial crisis(Van Boeckel*et al.,* 2014).

The post-surgical wound infection patient had significantly higher levels of tumor necrosis factor alpha, interferon-gamma, and interleukin-10 than the control group. For the first time in Nnewi, Nigeria, we demonstrate that MDR-bacterial infection causes an exacerbated inflammatory response of TNF, interferon gamma (a potent pro-inflammatory cytokine), and interleukin-10 (a potent anti-inflammatory cytokine) produced by activated macrophages, monocytes, and natural killer cells in the study population. Our findings are consistent with those reported in conditions such as MDR tuberculosis, acute organ dysfunction, and bacteremia, where inflammatory cytokines are significantly elevated(Schino*et al.,* 2023). Beig*et al.*(2023), found that MDR-TB patients had significantly higher mean levels of IL-10 and TNF-α compared to control subjects. The moderate increase in IL-10 response to MDR-bacterial isolates observed in these studies suggests that MDR-bacterial

infections reduced the patient's ability to effectively control the microbial infection. It had also shown that in patients with human immunodeficiency virus infection and other experimental infection have high levels of both pro and anti-inflammatory cytokines, which correlated with susceptibility to the pathogens and the severity of the associated disease (Naik *et al.,* 2022, Smialek *et al.,* 2022). However, studies have shown that exposing retinal and microglial cells to MDR bacterial strains results in increased levels of IL-6, IL-1α, IL-8, IL-10, tumor necrosis factor-alpha, and interferon-gamma (Naik *et al.,* 2021, Kany *et al.,* 2019, Mohan *et al.,* 2023). Thus, excessive inflammatory response observed in this present studymay impede infection resolution, which may help to explain in part, the poor treatment outcome in patients with MDR infections in Nnewi, Nigeria, even after prompt antibiotic treatment.Furthermore, understanding the drug resistance strategies possessed by the etiologic agents of surgical site infections will significantly improve chemotherapeutic approaches in the treatment of wound infections worldwide.

*Conclusion:*MDR-bacterial infections are more virulent, and the observed excessive inflammatory response may impede infection resolution, which may help to explain in part, the poor treatment outcome in patients with MDR infections, even after prompt antibiotic treatment.Thus**,** understanding the types of bacterial pathogens, antimicrobial resistance and cytokine patterns can help optimize treatment and reduce disease morbidity and mortality rates from surgical wound infections in Nnewi, Nigeria.

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