

Original Research Article

Multi Drug Resistance and Multi Antibiotics Resistance Index of *Acinetobacter baumannii* Isolated from Hospitals in Port Harcourt Metropolis

ABSTRACT

Acinetobacter species are aerobic gram-negative bacilli that can cause healthcare-associated infections and can survive for prolonged periods in the environment. Also on the hands of healthcare workers, infection due to *Acinetobacter* species is a major challenge within the health care facilities and the community in general due to their high drug resistance. The study was aimed at detecting multi drug resistance and multi antibiotics resistance index (MARI) of *Acinetobacter baumannii* isolated from hospitals in Port Harcourt metropolis in Rivers State, Nigeria. The cross sectional study sampled randomly; as urine and wound swab samples were collected from patients. *Acinetobacter* spp was isolated using standard microbiological methods. Identification of *A. baumannii* isolates were done using Phynotypic methods such as culture on Lead *Acinetobacter* medium and conventional biochemical tests. Antimicrobial susceptibility test was done by Kirby Bauer's disk diffusion method under Clinical Laboratory Standards Institute (CLSI, 2013) guide. Suspect *Acinetobacter* species were further identified using polymerase chain reaction (PCR) and Sanger sequence typing methods. The results of confirmatory sequence typing of isolates showed that 9 of suspect *Acinetobacter* spp were *A. baumannii*. The results of this finding showed presence of *A. baumannii* species resistant to conventional antibiotics. All isolates demonstrated MDR and XDR. MARI was high (>0.2) indicating MDR and high risk. This study established high rate of multidrug resistant *Acinetobacter baumannii*. There is need for improved sanitary working condition and proper patients' management to reduce the spread of this health care associated infection as well as a Search for new therapeutic alternative and policies to control the use of antibiotics.

Keywords: Drug, Resistance, Antibiotic, *Acinetobacter baumannii*

Introduction

Acinetobacter species are aerobic gram-negative bacilli that can cause healthcare-associated infections and can survive for prolonged periods in the environment and on the hands of healthcare workers [1]. *Acinetobacter* was first described in 1911 as *Micrococcus calcoaceticus* by Beijerinck, a Dutch microbiologist who isolated the organism from soil. Since then, it has had several names, nowadays known as *Acinetobacter* since the 1950s [2,3]. The genus *Acinetobacter* consists of more than 30 species, of which *A. baumannii*, and to a lesser extent genomic species 3 and 13TU, are mostly associated with clinical environment and nosocomial infections [2].

Acinetobacter baumannii is a Gram negative coccobacillus, aerobic, non-fermentative and non-motile bacterium that belong to the genus *Acinetobacter*. Current taxonomic classification of this bacterium put it in γ -proteobacteria, family Moraxellaceae and order Pseudomonadales [4]. It belongs to *Acinetobacter calcoaceticus-baumannii* complex group which comprises four different *Acinetobacter*: *A. baumannii*, *Acinetobacter pittii*, *Acinetobacter nosocomialis*, and *Acinetobacter calcoaceticus* [5, 4,6]. *Acinetobacter baumannii* has become increasingly responsible for causing hospital acquired infections (HAI), particularly in intensive care units (ICUs) [7]. It has been isolated from blood, sputum, skin, pleural fluid, and urine, usually in device associated infections [8]. The species are excellent biofilm producing bacteria, which facilitate their survival in hospital environments and are frequently found on the skin and in the respiratory and urinary tracts of hospitalized patients [9].

Antibiotics are the most active chemotherapeutics among drugs; they exert their therapeutic effect by antagonizing the growth of bacteria. [10]. Since 1910 many antibiotics have been developed with different mechanisms of action including: inhibition of bacteria cell wall synthesis; this class of antibiotics includes vancomycin and β -lactam antibiotics such as

penicillins, cephalosporins and carbapenems, inhibition of protein synthesis including tetracyclines, aminoglycosides, macrolides and chloramphenicol [10] and DNA synthesis inhibitors such as fluoroquinolones and sulfonamides that inhibit folic acid synthesis. [10]. Resistance happens when bacteria develop the ability to defeat the antibiotic designed to kill them [10]. The determination of antimicrobial susceptibility of a clinical isolate is often crucial for the optimal antimicrobial therapy of infected patients. This need is only increasing with increasing resistance and the emergence of multidrug-resistant microorganisms [10]

Literature has shown that the global burden of infections caused by *A. baumannii* still remains unknown due to the lack of comprehensive data especially from African countries [11], however, the burden can be up to 35% [12] with a mortality rate of 26% and this can increase to 45% in intensive care unit (ICU) [6]. In Nigeria like other African countries, the situation is not different, although Egwuenu *et al.* (2018) reported that, *A. baumannii* was associated with blood stream catheter-associated infection from different parts of the country including carbapenem-resistant *Acinetobacter* spp [13,14,15,16, 10,17]. *Acinetobacter baumannii* is one of the most challenging pathogens among ESKAPE pathogens, standing for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacteriaceae*, capable of "escaping" from common antibacterial treatments due to its particular antibiotic resistance [12]. The bacteria produce naturally occurring AmpC β -lactamases, as well as naturally occurring oxacillinases (OXAs) with carbapenemase activity [18].

Multi-drug resistance (MDR) in *Acinetobacter* species is defined as non-susceptible to at least 1 agent in ≥ 3 antimicrobial categories [19]. The species are becoming increasingly resistant to nearly all routinely prescribed antimicrobial agents, including aminoglycosides, fluoroquinolones, and broad-spectrum β -lactams. The majority of strains are resistant to the cephalosporin class of antimicrobials and resistance to carbapenems is increasingly reported [20]. Carbapenems which were once the mainstay therapy are no longer effective in controlling the infections caused by this organism. The foremost implication of infection with carbapenem-resistant *A. baumannii* is the need to use "last-line" antibiotics such as colistin, polymyxin B, or tigecycline [21]. Sulbactam, a β -lactamase inhibitor, has good in vitro activity against *Acinetobacter* species and has been used successfully for treating carbapenem-resistant strains [22].

Although studies have shown antibiotic resistance but few have been done in this region and fewer captured the Multiple Antibiotic Resistance Index (MARI) of *Acinetobacter baumannii*. The study investigated multidrug resistance and Multiple antibiotic resistance index of *Acinetobacter baumannii* isolated from selected hospitals in Port Harcourt metropolis, Rivers State Nigeria.

Materials and Methods

Study Area

The study area was carried out in Port Harcourt Metropolis in Rivers State. It was carried out in two tertiary hospitals in the state which are: The Rivers State University Teaching Hospital, (RSUTH), Port Harcourt, and University of Port Harcourt Teaching Hospital (UPTH). Rivers State is situated in the South-South region of Nigeria with a population of 5,198,716 according to 2006 Census report and is located at coordinates, 4° 55' N, 6°50'E. Rivers State is bound at the south by Atlantic Ocean, the North by Imo, Abia, and Anambra States, to the East by Akwa-Ibom State and to the West by Bayelsa and Delta States.

Study design

This was a cross sectional descriptive hospital based study which involved molecular characterization of *A. baumannii* isolated from urine and wound swab samples patients' who were eighteen years and above of ages and both sexes with prolonged hospital admission admitted in RSUTH and UPTH were recruited for the study. The isolated *A. baumannii* were characterised using both phenotypic, polymerase chain reaction (PCR) and Sanger sequencing methods.

Inclusion Criteria

Male and Female patients (18-35 years) who has not been on antibiotics in the last one month and agreed to participate in the study were included in this study.

Exclusion Criteria

Those who are on antibiotics and those who refuse to participate in the research were excluded.

Sample Collection

Clinical samples comprising of urine and wound swab were collected from the department of Medical Laboratory Science in RSUTH and UPTH, all within Port Harcourt.

Sample Processing

Isolation and identification of *A. baumannii*

All samples (both urine and swabs) were inoculated on freshly prepared MacConkey agar (HiMedia Laboratories Pvt Ltd, Mumbai, India, M173) media and incubated for 24 hours at 37°C. After incubation, isolates that were non lactose fermenting (shiny mucoid and tomb shaped) on MacConkey agar, Gram negative coccobacilli and oxidase negative were sub-cultured on Lead *Acinetobacter* Media (HiMedia Laboratories Pvt Ltd, Mumbai, India, M1839) and incubated at 37°C for additional 18-24 hours. Suspected *Acinetobacter* spp from Lead *Acinetobacter* Media (that is, pink colour) colonies were further identified using biochemical tests such as, catalase, coagulase, indole, citrate utilization, urea, and methyl red, [23]

Antibiotics Susceptibility Testing

Antimicrobial susceptibility test was done by Kirby Bauer's disk diffusion method under Clinical Laboratory Standards Institute (CLSI, 2013) guide. Five colonies of the organism were emulsified in 5mls of sterile normal saline and mixed well; the turbidity was compared to 0.5 Mac Farland standards.

A sterile cotton swab was used to inoculate the sample into Mueller Hinton Agar plates (Oxford, Cambridge UK) and allowed to dry. 15 antibiotics were placed on the plate. The plates were incubated overnight at 37°C and examined for inhibition of growth.

Zone of inhibition were determined by measuring the size of clear zones with a graduated ruler. The measurement was done in millimetres and the zones were compared with the CLSI standards for interpretation (CLSI, 2013). The reporting was done by indicating Resistant, Intermediate or Sensitive (CLSI, 2013).

Molecular identification of *A. baumannii*

DNA extraction (Boiling Method)

Overnight broth culture of the bacterial isolate in Luria Bertani (LB) transferred into 1.5ml eppendorf tube and was spun at 14000rpm for 3 min in a micro-centrifuge. The supernatant was discarded and 1000µl of 0.5% normal saline was added to the sediment and was vortexed on el tech XH-B vortexer. The cells were re-suspended in 500ul of normal saline and heated at 95°C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20°C for other downstream reactions.

DNA quantification

The extracted DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitres of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button. It was quantified using 5-100ng/µl at a purity of 1.5-2.0 if <5 it was repeated when >5 serial dilutions will be carried out.

Polymerase chain reaction

The 16s rRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40µl for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 52°C for 30 seconds; extension, 72°C for 30 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light trans-illuminator.

Sequencing

The purified PCR product was subjected to cycle sequencing using the BigDye terminator v3.1 cycle sequencing kit (Applied Bio systems, USA). Sequencing reactions were prepared as a 6µl reaction mix containing; BigDyeDirect Sequencing Master mix (2µl), sequencing primer (MP13Forward/ Reverseprimer) (1µl) and PCR product (3µl), and loaded 3µl of the reaction mix to the appropriate well in the respective forward or reverse reaction plate. Sequencing was performed in a thermocycler using the following conditions; at 96°C (1 min), followed by 25 cycles of 96°C (10 s), 50°C (5 s) and 60°C (75 s). At the end of the reaction, the tubes were briefly centrifuged and samples loaded onto the ABI 3700 gene sequencer. The results obtained were analyzed using MEGA software (version 6.0) and blastn (NCBI). A phylogenetic tree was constructed using Neighbour-Joining method and bootstrapping performed by creating 1000 trials. The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.11983619 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (200 replicates is shown next to the branches (Rzhetsky&Nei, 1992; Dopazo, 1994). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the

number of base substitutions per site. This analysis involved 20 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1610 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Ethical Approval/Informed Consent for the Study

Ethical approval for the study was gotten from the ethics committee of the Rivers State Ministry of Health. An informed, written consent was obtained from each participant after explanation of the purpose of the study and were assured of strict confidentiality. They were given the option of not participating in the study if they wanted.

Statistical Analysis

Statistical Package for Social Science version 21 was used for statistical analysis after collating data into Microsoft excel spread sheet. Frequency, percentage distribution were estimated. T test was used for parametric comparison of means of two groups while Kruskal Wallis was used to compare means of three groups for data not normally distributed. All deductions were made at 95% confidence level. P-value<0.05 was considered significant. Data presentation appeared on table as well as chart for data visualization.

Results

Evaluation of the Multi Drug Resistance (MDR) and MARI of *Acinetobacterbaumanni* isolated from Hospitals in Port Harcourt Metropolis in Rivers State Nigeria was performed using molecular method for identification as the confirmatory and the following findings obtained. Firstly, the study included sample size of 12 after initial scrutiny for the molecular assay and nine (9) isolates were confirmed *Acinetobacterbaumanni* of which these organisms were either MDR or XDR with high MARI. Samples where these microorganisms were obtained includes urine and wound swab from male and female subjects equally (50% each). Likewise, a proportionate distribution from the study sites - RSUTH and UPTH all 50% each. However, urine samples were more 83.3% compared to wound swab 16.7%. Details of these are shown on figure 1 to figure 3.

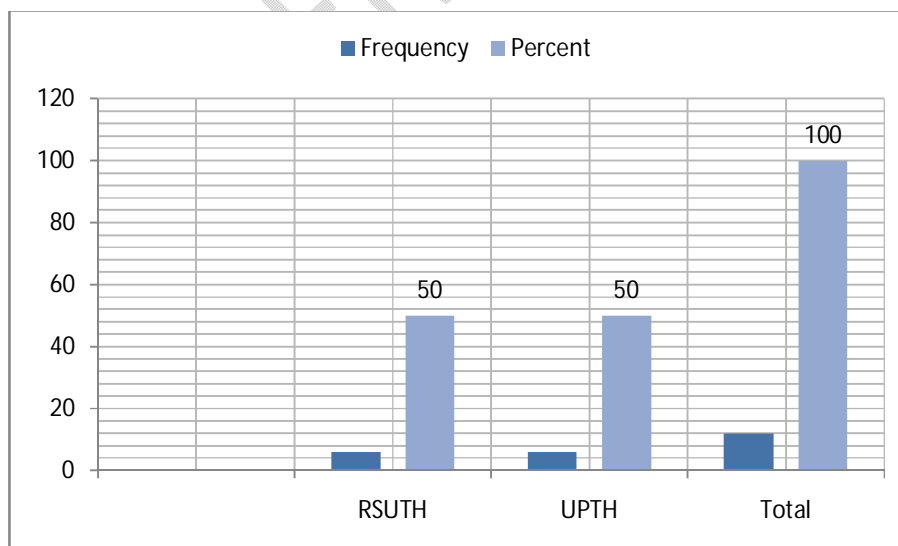


Figure 1: Frequency Distribution of Study Sites using Bar Chart

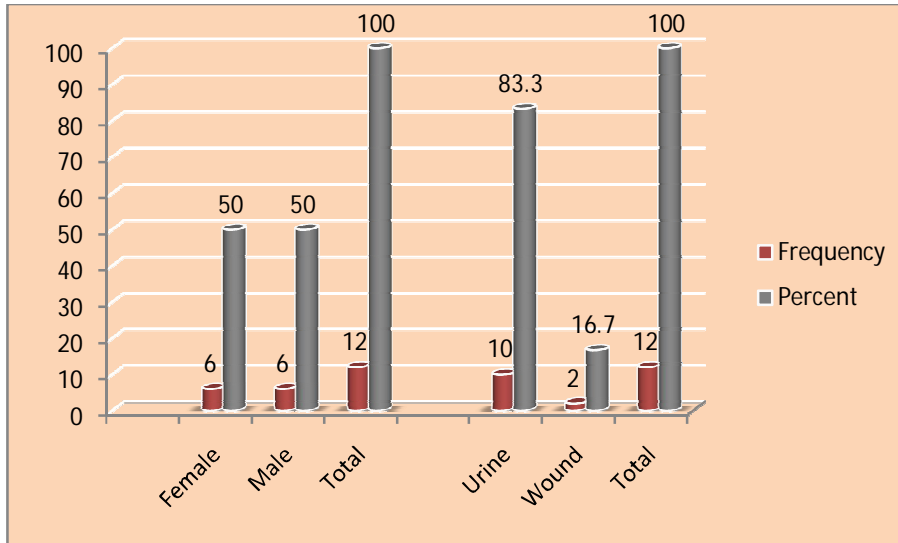


Figure 2: Frequency Distribution of Sex and Samples using Bar Chart

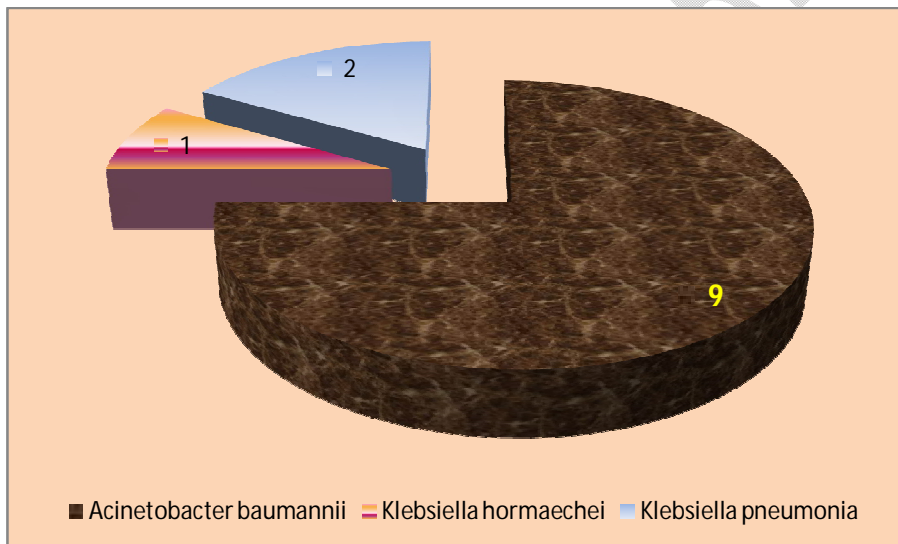


Figure 3: Frequency Distribution of Isolates

Multi Drug Resistance (MDR) of *Acinetobacterbaumannii*

Table 1 revealed the distribution Patterns of Multidrug Resistance in *Acinetobacterbaumannii* by Antimicrobial Class. The report of RSUTH location suggests 18 as the total number of resistant for 8 different categories of drugs with an overall MDR class of XDR. Although the different urine sample demonstrated different MDR classes as XDR, MDR, and MDR separately. Nevertheless, the UPTH location differ in observation. Forty-one (41) was the total number of resistant for 8 different categories of drugs. Furthermore, MDR classification revealed MDR, MDR, XDR, XDR, MDR, and MDR for 4 samples of urine and 2 samples of wound swab respectively. An overall MDR class for UPTH was XDR. Comparing the difference in the distribution, the study showed an indication of statistically significant difference using KruskalWallis (Chi Square = 36.428, Df =7, p=0.00).

Table 1: Distribution Patterns of Multidrug Resistance in *Acinetobacter baumannii* by Antimicrobial Class

Isolates		No. of Antimicrobials	Amioglycosides	Cephalosporins	Fluoroquinolones	Folate Pathway Inhibitors	Macrolides	Penicillins	Phenicol	Tetracyclines	Total Number Resistant	No. of Categories	MDR Class
RSUTH	Urine	15	-	3	-	1	1	1	1	1	8	6	XDR
	Urine	15	-	3	-	-	1	-	-	1	5	3	MDR
	Urine	15	1	-	-	1	1	1	-	1	5	5	MDR
	Total		1	6	-	2	3	2	1	3	18	8	XDR
UPTH	Urine	15	-	3	-	1	1	1	1	-	7	5	MDR
	Urine	15	-	2	-	-	1	1	1	1	6	5	MDR
	Urine	15	1	3	1	1	1	1	1	1	10	8	XDR
	Urine	15	-	2	-	1	1	1	1	1	7	6	XDR
	Wound	15	-	2	-	-	1	-	-	1	4	3	MDR
	Wound	15	-	3	-	1	-	1	1	1	7	5	MDR
Total			1	15	1	4	5	5	5	41	8	XDR	
Cumulative Total		15	4	21	1	6	8	7	6	8	59	8	XDR
Kruskal Wallis (Chi Square)					36.428								
Df					7								
p-value					0.00								
Remark					Significant								

MDR: Multi-Drug Resistance,

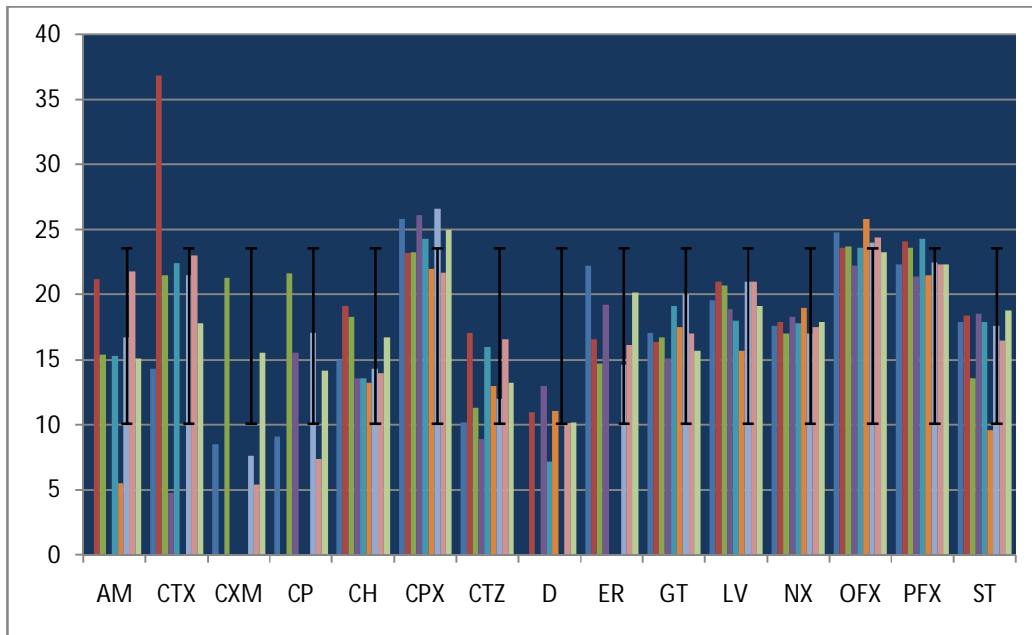


Figure 4: Multi Drug Resistance (MDR)

Multiple Antibiotic Resistance Indices(MARI)

The study illustrated the Multiple Antibiotic Resistance Indices of *Acinetobacterbaumannii*. Fifteen (15) drugs were tested and the results displayed varying degrees of resistance and Multiple Antibiotic Resistance Indices were extrapolated. RSUTH location had 8, 5, 5 and a total of 18 for resistance of the three samples of urine with MARI of 0.53, 0.33, 0.33 and 0.40 respectively. Notably, the study recorded an overall resistance of 59 with MARI of 0.44. Remarkably, if MARI is greater than 0.2 is a high risk environment, organisms living there are likely going to be multi drug resistant, but if MARI IS less than 0.2 is a low risk environment, organisms there's are not likely to be multi drug resistant. Base on this, the study recorded values greater than 0.2 hence high risk and mainly multidrug resistant organisms were isolate. See table 2.

Table 2: Comparison of Multiple Antibiotic Resistance Indices of *Acinetobacterbaumannii*

Location	Sample	No of Drugs Tested	Resistance	MARI
RSUTH	Urine	15	8	0.53
	Urine	15	5	0.33
	Urine	15	5	0.33
	Total	45	18	0.40
UPTH	Urine	15	7	0.47
	Urine	15	6	0.40
	Urine	15	10	0.67
	Urine	15	7	0.47
	Wound	15	4	0.27
	Wound	15	7	0.47

	Total	90	41	0.45
Overall Total		135	59	0.44

Further descriptive statistics showed $.3967 \pm .11547$ for Mean \pm SD for RSUTH. The UPTH location had results that did not differ statistically when compared to the RSUTH. $.4583 \pm .12968$ was reported for UPTH and mean comparison of the MARI from the two locations $t = -0.724$, $df = 7$, $p = 0.50$ with no significant discrepancy. See table 3, for detail.

Table 3: t test of Multiple Antibiotic Resistance Indices of *Acinetobacterbaumannii*

	RSUTH	UPTH	T test	Df	p-value
Mean	3967	.4583			
Standard Deviation	11547	.12968	0.724	7	0.50

Discussion

Nosocomial acquisition of multi-drug resistant *Acinetobacterbaumannii* has remained an issue of great concern. This is attributed to different factors including the enormous ability of *Acinetobacterbaumannii* to spread from along with colonization of human and environmental reservoirs [24]. This study evaluated Multi Drug Resistance and MARI of *Acinetobacterbaumannii* Isolated from Hospitals in Port Harcourt Metropolis in Rivers State Nigeria with a notable outcome of high Multidrug Resistance (MDR) of *Acinetobacterbaumannii* and Antibiotic Resistance Indices (MARI) of *Acinetobacterbaumannii*.

Detection of this organism in the samples used here is in conformity with Mayasari & Siregar [8]. Detection in these samples indicates colonization of some body systems and tracts such as the skin and urinary tracts. This finding is backed up by earlier research which illustrated the presence of this organism in hospitalized patients [9]. Also, this study confirms the work of Anitha & Monisha [7].

The study recorded massive distribution of multidrug resistant *Acinetobacterbaumannii*. Several evidences have been reported about the negative impact of this strain. Globally, it constitutes a clinical and public health menace especially to patients who are on therapeutic antibiotics. Study finding here showed that, the organism has developed resistance to major classes of antibiotics is backed up by different reports as revealed in other studies [27, 28]. This has contributed to the outbreaks of hospital acquired infection worldwide and there is an increasing concern about high antibiotic resistance with raised therapeutic failure. This has limited treatment options and there is serious advocate for newer therapeutic options to combat this global threat else, this might possibly bring the World closer to the end of the antibiotic era with *Acinetobacterbaumannii* compared to methicillin-resistant *Staphylococcus aureus* according to Nwadike, Ojide, & Kalu [10]. The study finding is in agreement with the report of Giamarellou et al. [25].

The MDR and XDR classes of resistance observed in the study including high values of MARI confirm an extensive *Acinetobacterbaumanii* resistance. Researchers have established that extensive antimicrobial resistance may probably partly be as a result of the microorganism's relatively impermeable outer membrane and its surrounding exposure to a huge reservoir of resistance genes [10]. Similarly, an independent study shared equal observation[26]. In this same vein, Maragakis and Perl in a study in 2008 revealed supporting fact to that regard.

Antibiotic drugs are known to have therapeutic effect on *Acinetobacterbaumanii*[10]. However, same drugs were mentioned by Nwadikeet *al.*[10], and revealed increasing resistance and the emergence of multidrug-resistant strains [10]. Several studies observed increasing MDR trend with morbidity and mortality burden [12, 6, 11, 13, 14, 15, 16]. Furthermore, several other scholarly findings are consistent with this [10, 17] observations. In addition, MDR reported here followed Magiorakoset *al.*[19] and increasing level [20, 21]. One major concern about this organism is the formation of biofilm providing the organism with adaptive capacity to survive adverse condition including hospital environments [9]. Therefore, a solution is needed to hunt this increasing trend and its consequences.

In general, the increasing rate of indiscriminate use of these antibiotics both in the hospital and over the counter have heightened the problem with majority of Multi drugs resistant organisms in circulation. The observed evidence reported in this study is a proof of high level of antibiotic resistance especially multi drug resistance and to an extent extra drug resistance. Information from this study should serve as a wake-up call to all and sundry and surveillance and public health intervention is highly needed to this effect in order to curb the devastating impact inherent.

Proposal

The following are therefore recommended from the pragmatic evidence observed in this study; there should be enhanced and sustained proper detection of organism as well as improved sanitary working condition not neglecting proper patients' management to reduce the spread of this health care associated infection. Also, consideration should be made in the area of standardized methods of AMR testing prior to prescription and use. Besides, search for new therapeutic alternative and policies to control use of antibiotics and hospital-acquired infections should be a constant habit for Health Care Practitioners.

Limitation

The following were encountered as limitations in the course of the study: Difficulty in sample collection as patients delayed in giving their informed consent. The encountered delay in getting ethical approval from the designated authority. Further molecular characterization of *Acinetobacter* exploring the genes responsible for MDR was not performed. All strains were not tested against carbapenem due to lack of supplies.

Conclusion

Multidrug resistance profile showed evidence of multidrug resistant of *Acinetobacterbaumanii*(MDR and XDR) based on MDR classification. Also, Multiple Antibiotic Resistance Indexes of the *Acinetobacterbaumanni* isolates indicated high risk as all values of MARI were greater than 0.2. This confirmed the presence of multidrug resistant organisms in the study

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