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PLASMID MEDIATED RESISTANCE IN MULTIDRUG RESISTANT BACTERIA ISOLATED FROM CHILDREN WITH SUSPECTED SEPTICAEMIA IN ZARIA, NIGERIA

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ABSTRACT
Septicaemia is a common cause of morbidity and mortality among children in the developing world. The knowledge of the epidemiological and antimicrobial pattern of common pathogens that cause septicaemia is useful for prompt treatment of patients. Fifty-five (55) clinical isolates from children with suspected septicaemia were used for the study. The isolates include Coagulase negative Staphylococcus, Staphylococcus aureus, Salmonella spp., Klebsiella spp., Citrobacter spp., Proteus spp and Pseudomonas spp. The antibiotic susceptibility testing of isolated bacteria associated with septicaemia in children were carried out using standard microbiological protocol. The MAR index for the test bacterial isolates was determined and the bacterial isolates that displayed multiple antibiotic resistance were investigated for the presence of resistant factor such as plasmids. The sizes of the plasmid observed in the bacterial isolates were determined using agarose gel electrophoresis. Observations made from the agarose gel electrophoresis showed that majority of the multiple antibiotic resistant isolates haboured plasmids DNA of different sizes viz: 10.00 Kb, 8.71 Kb, 7.08 Kb, 1.02 Kb, 1.00 Kb, 0. 98 Kb and 0.87 Kb. The plasmid analysis of the results obtained in this study showed that the predominant plasmid molecular size was 977bp which occurred frequently among the Citrobacter spp and Staph aureus. These findings suggest an increased resistance to the antibiotics commonly used for the treatment of septicaemia, and the observed presence of plasmids in some of the test bacteria isolated shows that they could have been acquired from multiple antibiotic resistant bacteria in the community under investigation.
Key words: Children, Multiple antibiotic resistance, Plasmids, Septicaemia

INTRODUCTION

Septicaemia remains a major cause of morbidity and mortality in children. In the developing countries they occur more often among neonates and young infants and are mainly community acquired (Alausa *et al.,* 1977). While in the advanced countries they occur more often among elderly patients of which majority are hospital acquired following instrumentation and therapeutic procedures (Brunfitt and Leigh, 1969). The widespread use of antimicrobial agents against the high level of microbial infection in children is becoming a cause for increased concern. Resistant bacteria population flourishes in areas of high antimicrobial use, where they enjoy a selective advantage over susceptible population (Gordon and Ronald, 2005).

Frequently, a bacterial pathogen is drug resistant because it possesses plasmid that has one or more resistant genes. Plasmids have the ability to be transferred within and between species and can therefore be acquired from other bacteria. This property makes resistance due to plasmid much more threatening than resistance due to chromosomal mutation in terms of spread of antibiotics resistance (Hugo and Russell, 2004).

This study was carried out to investigate the antibiotic susceptibility pattern of bacteria isolates from children with suspected septicaemia in Institute of Child Health, Banzazzau, Ahmadu Bello University Teaching Hospital (ABUTH) Zaria and to assess whether antibiotic resistance in the isolated bacteria is plasmid or chromosomally mediated. It is hoped that with the findings from this study will give a guide to proper management septicaemia especially in upholding the existing empirical treatment of septicaemia, prior to obtaining culture results in a setting where there is limited resources and laboratory facilities for diagnosis and controlled use of antibiotics to prevent or minimize spread of antibiotic resistance to the locality.

MATERIALS AND METHODS

Fifty-five (55) clinical isolates specifically bacteria from children with suspected septicaemia were used for the study. The isolates include Coagulase negative staphylococcus, *Staphylococcus aureus, Salmonella* spp*, Klebsiella* spp*, Citrobacter* spp*, Proteus* spp *and Pseudomonas* spp. Antibiotic susceptibility pattern was determined using Kirby Bauer disc diffusion method. Overnight nutrient broth cultures which had been standardized with 0.9% Nacl solution to contain10^5 CFU/ml were inoculated on the surface of

the agar and allowed to dry ,after which the antibiotics were placed aseptically. This was allowed to stay for 30 minutes to allow diffusion of antibiotics into medium, and then incubated at 37^{o}C for 24 hrs.

Determination of Minimum Inhibitory Concentration

The MIC was determined using eight antibiotics: cefuroxime, ceftriaxone, ciprofloxacin, ofloxacin, gentamicin, erythromycin, streptomycin and chloramphenicol, employing the agar plate dilution method (Lennette *et al*., 1990) with modifications. Freshly prepared stock solution of the various antibiotics were prepared in graded concentration (0.7812 – 500µg/ml) of 5ml volume in triplcates and mixed aseptically with 5ml volume of double strength sterile Mueller Hinton's agar and allowed to set. Five (5) ml of sterile de-ionised distilled water mixed with 5ml double strength Mueller Hinton agar were set up as control. An 18 hours culture of the test isolates were standardized to an inoculum density of 10^{5}cfu/ml (Woods and Washington, 1995). The dried agar surface was aseptically inoculated with test organism in triplicates at equidistance. A positive control Mueller Hinton agar was inoculated with 20µl of the standardized over night-culture of test isolates (10^{5}cfu/ml). The plates were allowed to stay for 30 minutes after which they were incubated at 37^{o}C for 18 hours (Odama, 1990). They were thereafter examined for the presence or absence of growth. The lowest antibiotic concentration at which they were no visible growth was taken as the minimum inhibitory concentration (MIC). Peak blood plasma level as stated by Martindale (1996) was used as the break point.

MULTIPLE ANTIBIOTIC RESISTANCE INDEX The MAR index for the test bacterial isolates was determined according to the procedure described by Krumperman (1983). The indices were determined by dividing the number of antibiotics to which organisms were resistant (a) by the number of antibiotics tested (b). Resistant to three (3) or more antibiotics is taken as MAR.

Beta-Lactamase Production Test Suspension of the isolates was prepared by emulsifying bacterial colonies (from overnight cultures) in 0.5ml phosphate buffer solution containing 0.06mg/ml penicillin G. They were incubated at room temperature for at least an hour. Thereafter, 2 drops of freshly prepared 1% aqueous starch solution were added to each bacterial suspension and shaken. Then, one drop of iodine solution was added and allowed to stand for ten minutes. (Olayinka *et al*., 2005).

Isolation Of Plasmid DNA Sterile Luria-Bertani (LB) medium was inoculated with a single bacterial colony and incubated at 37^{o}C for 24 hours to form a good growth to saturation. Exactly 1.5ml of cells was centrifuged for 1 minute at 8000rpm. Pellets were re-suspended in 400 µl Glucose/Tris/EDTA (GTE) solution and allowed to stand for 5 minutes at room temperature. Two hundred (200) µl of NaOH/SDS (Sodium hydroxide/Sodium dodecyl solution), was added and mixed well then placed on ice for five minutes. One hundred and fifty **(**150) µl of potassium acetate solution, was added and vortexed briefly and placed on ice for 5 minutes. This was centrifuged at 10,000 rpm for 5 minutes and supernatant transferred to a new tube. Eight hundred (800) µl of 95 % ethanol was added and minutes and supernatant decanted. Pellets were washed with 1ml 70% ethanol, centrifuged at 8000 rpm for 5 minutes and supernatant decanted. The ethanol was air-dried and pellet re-suspended in 50µl of Tris/EDTA (TE) buffer (Rederick *et al.,*1992).

Agarose Gel Electrophoresis of Plasmid DNA Agarose gel (0.8 %) was made by weighing 0.8g of agarose in a conical flask and 100 ml of 1 x TAE (Tris-acetate 0.002M Ethylene diamine tetra acetic acid) buffer was added. This was solubilized by heating in a microwave oven and allowed to cool to 60 ^{0}C thereafter 5µl of ethidium bromide was added. It was then sealed by tape on edges with thumb nail in gel tray and comb was placed in position. The combs were placed near edge of gel and made sure that fingers of comb are slightly above the plate but not touching it. The agarose solution was placed into taped gel tray to make gel. Three hundred (300) ml of 1 x TAE buffer for electrophoresis tank was prepared and transferred to a clean electrophoresis tank. The gel slab was placed horizontally in the electrophoresis tank and the comb removed gently. The gel was then totally submerged in buffer but not covered by more than 1cm above the gel. Five (5) µl of gel loading buffer was added to 15µl of each sample (total 20 µl) and the samples were carefully loaded in individual wells. The set up was then run at 75 volts for 45 minutes. After the dye front has travelled approximately 80% of the gel length, the gel was removed from the gel tray. Ethidium bromide stained bands in the gel were then photographed using a trans-illuminator (Rederick *et al.,* 1992).

Results

About 55 bacterial isolates from children with suspected septicaemia were screened for their drug resistant pattern, percentage susceptibility profile of the bacterial isolates by zone of inhibition shows that most of organisms were 100% sensitive to ceftriaxone except *Citrobacter* and *Proteus,* the gram-positive organisms were generally resistant to erythromycin and cefuroxime (Tables 1&2). Susceptibility of the test bacterial isolates to eight antibiotics using the peak plasma level showed that all the isolates were 100 % susceptible to ceftriaxone with the exception of *Citrobacter* (50 %) and *Proteus spp* (0%) to the test antibiotics (Table 3). The MAR indices showed that the least index was 0.3 while the highest was 0.8 (table 4). All the MAR bacterial isolates were found to produce β lactamase enzyme and were observed to be resistant to the β lactam antibiotics used in this study (Table 5). Agarose gel electrophoresis of plasmid DNA of the MAR isolates revealed the presence of plasmids. About 2(two) of the MAR isolates has 2 plasmids, while others have only one. The sizes of the plasmid DNA include 10 kb, 8.71kb, 7.08kb, 1.023kb, 1kb, 0.97kb, and 0.87kb (Fig 1&2). Fig. 3 shows semi-logarithmic graph of the molecular weight of the standard DNA marker vs the distance travelled by the DNA on the gel, this was used to estimate the molecular weight of the isolated plasmid DNA. Table 6 shows agarose gel electrophoresis of plasmid DNA of bacteria isolates with plasmid number and their approximate sizes.

Table 1: Percentage sensitivity profile of gram positive organisms isolated from children with suspected septicaemia in the Institute of Child Health A. B. U.T. H., Zaria by zone of inhibition.

Antibiotics	*Staphylococcus aureus* (%)	*CoNS* (%)
Co-trimoxazole	50.00	50.00
Gentamicin	45.83	75.00
Ciprofloxacin	0.00	50.00
Streptomycin	29.16	50.00
Erythromycin	0.00	0.00
Cefuroxime	0.00	0.00
Ceftriaxone	100.00	100.00
Ampiclox	4.17	0.00

Table 2: Percentage sensitivity profile of gram negative organisms isolated from children with suspected septicaemia in the Institute of Child Health A. B. U.T. H., Zaria by zone of inhibition.

Antibiotics	***Salmonella* species (%)**	***Pseudomonas* species (%)**	***Citrobacter* species (%)**	***Klebsiella* species (%)**	***Proteus* sp (%)**
Co-trimoxazole	69.23	50.00	100.00	100.00	0.00
Gentamicin	84.61	100.00	50.00	100.00	0.00
Ciprofloxacin	15.38	0.00	0.00	0.00	0.00
Streptomycin	69.23	100.00	50.00	100.00	0.00
Augmentin	38.46	100.00	50.00	0.00	0.00
Ofloxacin	84.61	100.00	50.00	0.00	100.00
Chloramphenicol	15.38	0.00	50.00	0.00	100.00
Ceftriazone	100.00	100.00	50.00	100.00	0.00
Cefuroxime	0.00	0.00	0.00	0.00	0.00

Table 3: Percentage Antibiotic sensitivity profile (using peak plasma level) of bacteria isolated from blood samples of children with suspected septicemia, Institute of Child Health, A.B.U.T.H Zaria.

Name of org.(No. of isolates)	GN (%)	ST (%)	OFX (%)	CP X (%)	CHL (%)	CXM (%)	RP (%)	ER
Staph aureus (24)	41.66	29.16	41.66	0.00	12.50	12.50	100.00	0.00
Coag-ve *Staph*(4)	50.00	50.00	75.00	75.00	0.00	0.00	100.00	0.00
Citrobacter spp (6)	50.00	50.00	50.00	0.00	50.00	0.00	50.00	-
Klebsiella spp(2)	100.00	100.00	0.00	0.00	0.00	0.00	100.00	-
Salmonella spp(13)	84.61	69.23	84.61	15.38	15.38	0.00	100.00	-
Proteus spp(2)	0.00	0.00	100.00	0.00	0.00	0.00	0.00	-
Pseudomonas spp(4)	100.00	100.00	100.00	0.00	0.00	0.00	100.00	-

Key: GN- Gentamicin; OFX – Ofloxacin; CHL- Chloramphenicol; RP- Ceftriaxone; ST- Streptomicin; CPX- Ciprofloxacin; ER- Erythromycin; CXM- Cefuroxime.

Table 4: Multiple Antibiotic Resistance (MAR) Indices of Bacteria Isolated from children with suspected septicaemia in Institute Child Health, ABUTH

No. of antibiotics to which org. are resistant	MAR Index	Frequency of MAR index						
		S. aureus (n = 24)	CoNS (n = 4)	*Salmonella Spp* (n = 13)	*Pseudomonas Spp* (n = 4)	*Citrobacter Spp* (n = 6)	*Proteus Spp* (n = 2)	*Klebsiella Spp* (n = 2)
	0.1	0	0	0	0	0	0	0
	0.2	0	0	0	0	0	0	0
3	0.3	0	0	1 (7.7)	0	0	0	0
4	0.4	2 (8.3)	1(25.0)	7(53.9)	4 (100.00)	0	0	0
5	0.5	1(4.2)	2(50.0)	0	0	1(16.7)	0	0
6	0.6	9 (37.5)	0	5 (38.5)	0	4 (66.7)	0	2 (100.0)
7	0.7	4 (16.7)	1(25.0)	0	0	1 (16.7)	0	0
8	0.8	8 (33.3)	0	0	0	0	2 (100.0)	0
9	0.9	0	0	0	0	0	0	0
	1.0	0	0	0	0	0	0	0

The MAR indices showed that the least index was 0.3 while the highest was 0.8.

Table 5: β- Lactamase production by resistant bacteria isolated from blood of children with suspected septicaemia in the Institute of Child Health, A.B.U.T.H. Zaria

Bacterial isolates	Number	β - lactamase production
Staphylococcus aureus	3	3
Coagulase neg.Staph	3	3
Citrobacter sp	2	2
Klebsiella sp	1	1
Salmonella sp	3	3
Proteus sp	1	1
Pseudomonas sp	2	2

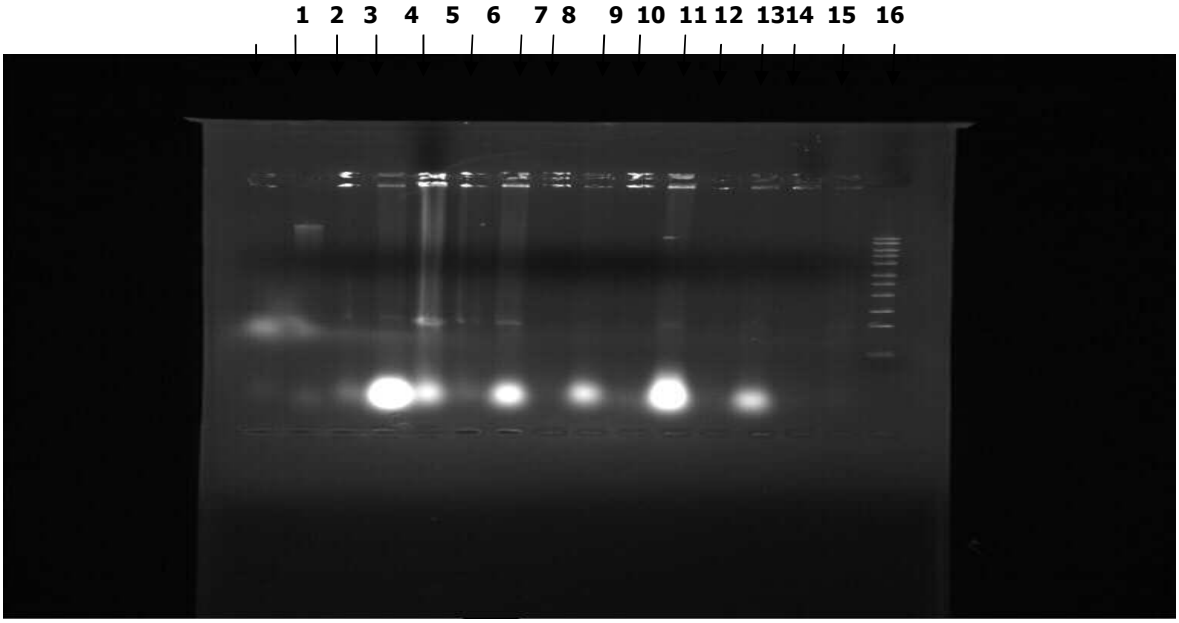

Fig 1 0.8% Agarose gel electrophoresis of plasmid DNA from MAR bacteria isolated from children with suspected septicaemia. Lane 1 - 7, lane 11, and 16 correspond with isolate laboratory numbers 8, 10, 45, 51, 53, 60, 63, 126, and DNA ladder, showing fragment sizes of 10. 00 Kb; 8.00 Kb; 6.00 Kb; 5.00 Kb; 4.00 Kb; 3.00 Kb; 2.50 Kb; 2.00 Kb; 1.50 Kb; 1.00 Kb and 0.50 Kb

↓9 15

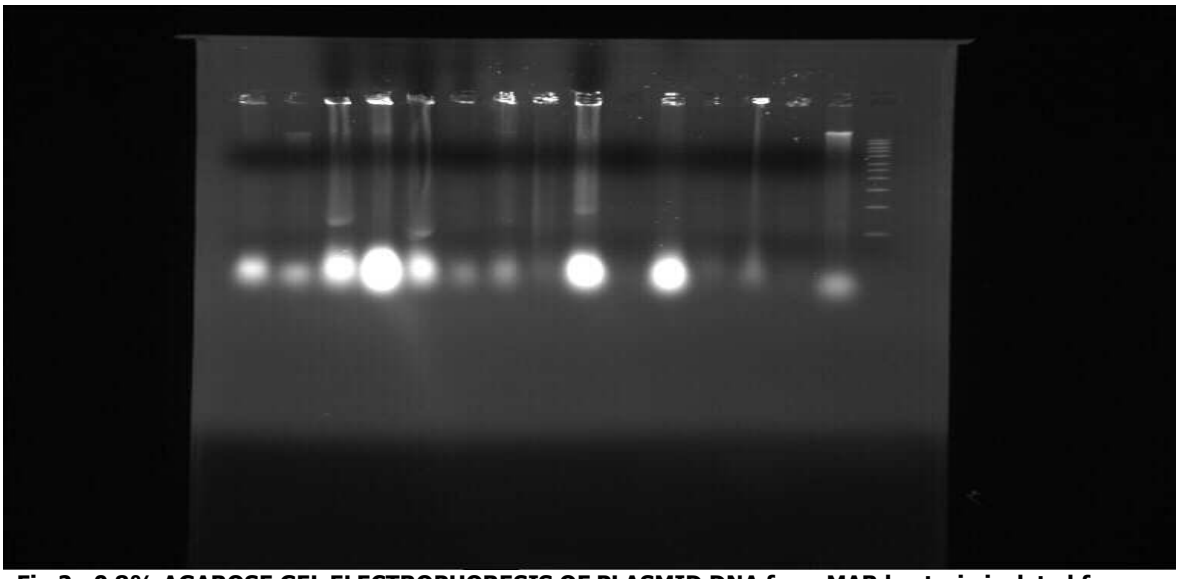

Fig 2: 0.8% AGAROSE GEL ELECTROPHORESIS OF PLASMID DNA from MAR bacteria isolated from children with suspected septicaemia. Lanes 9 and 15 has lab no. 102 and 135.

Table 6: Standard molecular weight sizes of the DNA marker

Molecular sizes of standard DNA ladder (Kb)	Log of molecular size of the marker	Distance moved by the band (cm)
10.00	4.00	1.70
8.00	3.90	1.90
6.00	3.78	2.00
5.00	3.70	2.10
4.00	3.60	2.20
3.00	3.48	2.30
2.50	3.40	2.60
2.00	3.30	2.80
1.50	3.18	3.10
1.00	3.00	3.50
0.50	2.70	4.10

Table 7: Agarose gel electrophoresis of plasmid DNA of bacteria isolated from suspected septicaemia patients showing plasmid number and their approximate sizes.

S/No	Isolate No.	Bacterial species	Plasmid No.	Approximate plasmid size (Kb)
1	8	*Citrobacter* spp	1	0.97
2	10	*Klebsiella* spp	2	8.71 and 0.87
3	45	*Citrobacter* spp	1	1.023
4	51	*Salmonella* spp	1	1.023
5	53	*Proteus* spp	1	1
6	60	*Salmonella* spp	1	1
7	63	*Staph. aureus*	1	0.97
8	126	CoNS	2	7.08 and0.87
9	102	*Staph.aureus*	1	0.97
10	135	*Pseudomonas* spp	1	10

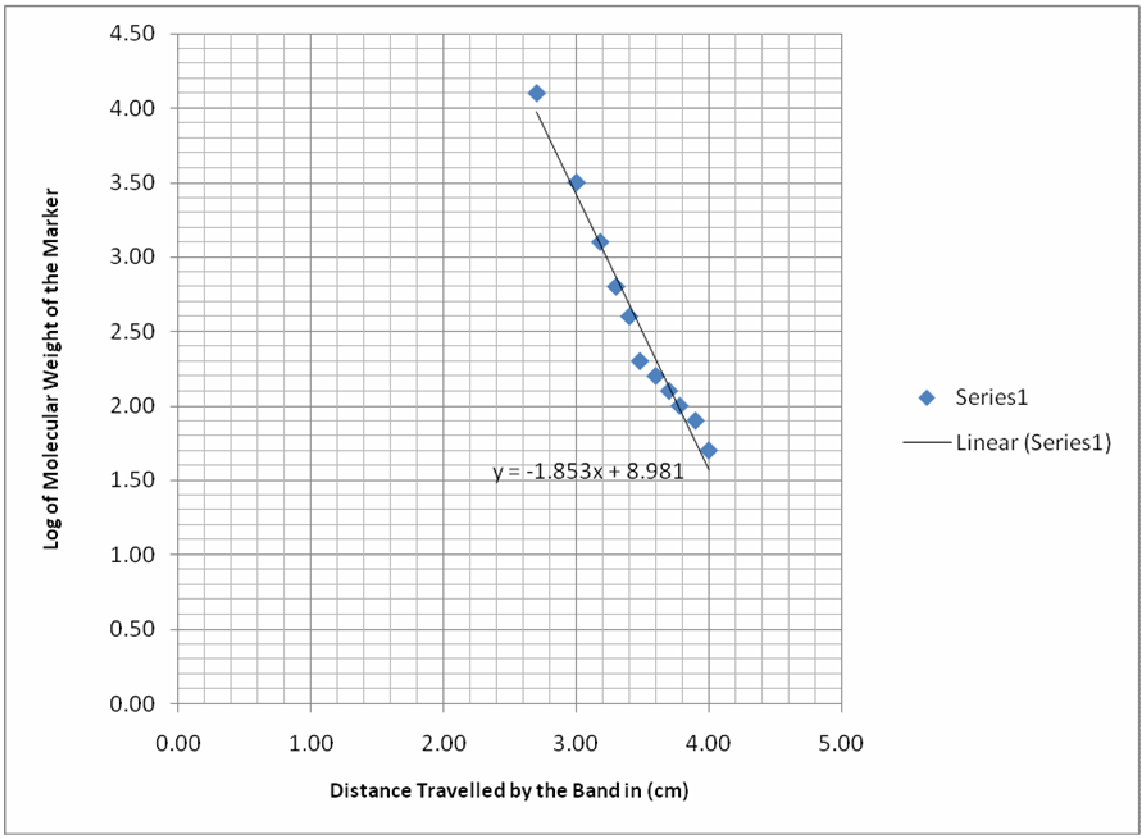

Fig 3: Semi-logarithmic graph of the molecular weight of the standard DNA marker vs the distance travelled by the DNA on the gel.

DISCUSSION

The extent to which bacteria develop resistance to antimicrobial drugs and the speed with which they do so vary with different types of drugs. New resistance mechanisms are emerging and spreading globally, threatening the ability to treat common infectious diseases resulting in prolonged illness and death (WHO, 2016) and a number of factors have been reported to be responsible for the spread of antimicrobial resistance genes among bacterial population plasmid mediated being one of the most significant (Huang *et al*, 2012). The test bacterial isolates in this study displayed high level of multiple antibiotic resistance to some of the test antibiotics. The MAR index determined in this study showed that the test isolates had values equal to and higher than 0.3.This shows that the test isolates may have come from environment where antibiotics are often used. Since the blood samples used in this study were from out-patients in the community, it could be concluded that there is widespread indiscriminate use of commonly prescribed antibiotics in the locality investigated.

The high level of resistance to the β -lactam antibiotics correlates with the ability of these isolates to produce β -lactamase enzyme. All the MAR isolates screened were found to produce β -lactamase . Such resistance due to β-lactamase producing bacteria (BPLB) have been reported in clinical isolates (Fashae *et al.,* 2004; Shukla *et al*., 2004; Siu *et al*., 1999; Hugo and Russell, 2004).The development of resistance to penicillin in *Staph aureus* by the production of β-lactamase quickly decreases the usefulness of penicillin for serious infections especially among hospitalized patients in whom resistant strains are frequently found Murray and Moellering, (1978). Studies have shown that plasmid bearing the gene encoding these β-lactamases frequently carries gene encoding resistance to aminoglycosides, chloramphenicol, doxycycline and co-trimoxazole (David and Roberts, 2005; Jacoby *et al*., 2002, Qin *et al*, 2008). Emergence of β lactam associated resistance in children is of seious concern as this class of antibiotic is often used as first line therapy for critical illness (Qin *et al*., 2008).

This is of serious concern as even the broad spectrum antibiotics such as cefuroxime and Augmentin® which are widely used empirically for the treatment of septicaemia are included as observed in this study. It has been reported that BLPB may not only survive penicillin therapy, but can also protect other penicillin susceptible bacteria from penicillin by releasing the free enzyme into their environment.

Agarose gel electrophoresis analysis in this study revealed the presence of plasmids in a majority of the multiple antibiotic resistant isolates tested. The molecular sizes of the plasmid DNA observed in this study were 10 kb, 8.71kb, 7.08kb, 1.023kb, 1kb, 0.97kb, and 0.87kb. Two (2) of the MAR isolates (*Klebsiella* sp and CoNS) have two plasmids, with size of 8.71kb, 0.87kb and 7.08kb, 0.97kb respectively. These organisms were simultaneously resistant to 3 to 4 antibiotics. Reports from some studies have shown that *Klebsiella* exhibit resistance to multiple drugs, even to the structurally unrelated antibiotics and it habours plasmids that are capable of disseminating drug resistance to other bacterial population (Gutmann *et al*., 1985; Sanders, 1984; Rasool *et al.,* 2003). The plasmids found in CoNS may have been acquired from the environment as a result of selective pressure. It has also been reported that CoNS is capable of disseminating antibiotic resistance in the community and habours chloramphenicol, erythromycin, streptomycin resistance gene (Kessie *et al.,* 1998).

Pseudomonas spp isolated in this study was found to harbor a single plasmid of molecular weight 10 kb. *Pseudomonas aeruginosa* has been reported to have different plasmids that mediate resistance to third generation cephalosporins and penicillins through the β lactamase enzymes (Jafari *et al,* 2013), it has also been found to habour plasmids such as pbs12 and pbs31 which account for a significant level of resistance of *Ps*. *aeruginosa* especially when exposed to UV irradiation (Anisimova, *et al.,* 1982). Studies have shown that accumulation of resistance factors have rendered *Staph aureus* immune to a variety of commonly used antibiotics, thus increasing its ability to survive in hostile environs. *Staph aureus* resistance have been shown to be as a result of acquisition of mecA gene (Neu,1992) especially to a variety of antibiotics whose mechanism of action are similar to β-lactam antibiotics, as observed in this study. The 2 multiple antibiotic resistant *Citrobacter* sp used in this study showed the presence of plasmids with 0.97bp and 1.023kb. A study has shown that *Citrobacter freundii* strains habour plasmids that carry one of β-lactam gene of the TEM family which inactivates such drugs as the ampicillin and extended spectrum cephalosporins (Janda, 2006), this bacteria has also been reported to carry qnrA and qnrB gene which were transferrable (Yang *et al*,2008). These findings suggest an increasing rate of resistance to some commonly used antibiotics in the treatment of septicaemia, further studies should be carried out on plasmid profiling this will help in epidemiological surveillance of disease outbreaks and also detecting antibiotic resistance pattern.

In conclusion, this study shows the presence of multiple antibiotic resistant isolates which were quite high in children with suspected septicaemia, it also showed that majority of the MAR isolates harboured resistance factor i.e. R-plasmids which probably must have been acquired from MAR resistant bacteria in surrounding environment. Therefore there is a need to monitor and restrict the use and sale of antibiotics to only conditions that require it, this will help reduce the spread resistant factors in the environment, if not we may be challenged in near future of having no useful antibiotics for the treatment of severe and life threatening infections such as septicaemia.

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Contribution of authors: AbdulAziz ZA carried out the laboratory work, Ehinmidu JO, Conceived the idea, Ehinmidu JO and Adeshina GO supervised the work, Bugaje MA assisted in the collection of isolates, Yusuf SS and Pala YY assisted in the plasmid analysis. All authors participated in literature search and the write-up.

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