PLASMID PROFILING AND CURING ANALYSIS OF MULTIDRUG RESISTANT ISOLATES FROM NEONATAL BLOOD CULTURE IN TERTIARY INSTITUTION

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Abstracts: This study seeks to determine the plasmid DNA profile of multidrug resistant organisms isolated from neonatal blood culture. The study design was prospective and specimens were collected in a specified order from neonatal intensive care unit (NICU). Neonates that were both in-born and out-born were used in this study. Specimens were collected from three hundred babies out of four hundred babies that were admitted into the neonatal intensive care unit (NICU) of the Hospital. One milliliter of blood was aseptically inoculated into each blood culture bottle of brain heart infusion broth and thioglycollate broth, they were incubated aerobically at 37ºC for up to seven days, and examined daily for bacteria growth using turbidity, bubbles and gas productions as an indication of presence of bacteria. Samples were cultured on MacConkey, Chocolate and Blood agar. Clinical isolates were identified to species level using the protocol of Cowan and Steel method. Antibiotic Susceptibility Testing (AST) was done using dilution method described by Fleming. Mueller Hinton Broth (MHB) was used as the diluents. Clinical isolates were subjected to Plasmid DNA profiling and curing test was carried out on multidrug resistant using ZymoPURE Plasmid Miniprep. This was followed by a post Plasmid curing test using Sodium Deodecyl Sulphate (SDS). 0.8% Agarose gel electrophoresis was carried out to separate the Plasmid DNA using 5µl ethidium bromate dye. Bands were visualized using ultra violet (UV) illuminator. Isolates that were multi-resistant yielded one or more plasmids. Plasmids curing made Pseudomonas aeruginosa, Proteus mirabilis and Klebsiella pneumonia that were formerly resistant to become susceptible. These findings suggest that environmental factors and genetic make- up of bacteria are important determinants of organisms' susceptibility pattern.

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1. INTRODUCTION

The increase in the rate of multidrug resistant in our current world has post a great deal of public health challenges to our health Institution. The causes of this antibiotic resistant varied in different dimensions which are not quite clear (CDC, 2013).

The continuous and rapid increase in difficulties in treatment of infections has become a major problem to deal with (Visuwamathan, 2014; Alanazi, *et al*., 2018). According to Joshi, 2017, in 30 years' time over 10 million people may likely died from difficulties to treat infectious diseases due to resistant (Tima, 2018). Some Scientists have tagged this era, as **"post antibiotic era"** (Michael *et al*, 2014). One of the causes of this antibiotics resistance is Plasmid acquired bacteria and this has triggered higher morbidity and mortality rate (Finley et al, 2020).

Plasmids confined a selective advantage on organisms that carried them such as resistance, to antibiotics, heavy metals, and also produce antibiotics that enable the host to compete for food and space (Savara and Rankin, 2011; Liu *et al.,2016;* Poirel *et al.,* 2016; Ridenhour *et al.,* 2017).

There are various types of plasmids and they are useful in different ways (Wang *et al,*. 2011). There are **virulence plasmids**: which enables the organism to become more pathogenic through production of toxins. **R-factor:** confers ability to develop resistant against antibiotics on the organism, thereby making it difficult to treat.

Cell resistant factor: **F-Factor**, this helps in fertility factor during Conjugation (Wang *et al*., 2011; Rossolini *et al*., 2014).

2. METHODOLOGY

The study was carried out in neonatal intensive care unit (NICU) in Delta State University Teaching Hospital (DELSUTH), Oghara. It is a few kilometers from Benin City Edo State, and about two hours journey from the State Capital Asaba. The Facility is a Quaternary Institution that serves the neighboring States, such as Edo and Bayelsa.

This study was done using 300 babies samples collected from NICU Department of the Hospital. The samples were collected over a period of time due to the nature of the samples being restricted only to neonates**.**

We got Ethical approval from the Ethic and Research Committee of the Institution following the research proposal submitted to the Committee.

The neonate blood samples were collected and inoculated into the blood culture media brain heart infusion broth (BHI) and thioglychollate broth in ratio 1:10 each of the media. They were incubated for seven days at 37^{o}C while they were daily examined for signs of bacterial growth such as production of gas bobbles or turbidity. Blood culture media found with the sign(s) of bacterial growth were subculture into Chocolate, Blood and MacConkey agar while Thioglychollate broth was subculture into blood agar only for the purpose of isolation of anaerobic organisms. They were all incubated at 37^{o}C for 24 hours, and all Clinical isolated were identified to species level using the method of Clinical and Laboratory Standards Institute (CLSI, 2015).

Antibiotic susceptibility test (AST)

Dilution method of antibiotic susceptibility described by Alexander Fleming and was used. The isolates were suspended in Mueller Hinton broth (MHB) and the suspensions were made to match 0.5 McFarland Standard. Standard antibiotics that are currently being used were placed on the plates. The antibiotics and their concentrations include: Ofloxacin 5µg, Augmentin10µg, Cefuroxime30µg, Erythromycin 25µ, Gentamicin 10µg, Cefotaxime 10µg, and Ceftriaxone 10µg. (CLSI 2015).

PLASMIDS DNA PROFILING

Those bacteria that were multi-drug resistant were subjected to plasmids DNA profiling and curing. The process involved three stages which included: plasmids extraction, preparation of agarose gel and running of the gel CLSI, (2019).

The extraction of the plasmid was done by using ZymoPURE reagent (Zaman*et al.,* 2010).

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was carried out on the harvested plasmids using 0.8% w/v agarose gel. The gel was loaded at 120V for 42minutes and viewed under Ultra Violet (UV) trans illumination and photographed. The approximate molecular weight (m.wt) of each plasmid was consequently obtained by extrapolation on graphical plots of molecular weight of marker against the distance traveled by the respective band.

PLASMIDS CURING

When applied to genetic materials such as plasmid DNA, the term curing refers to the treatment that promotes the loss of resident plasmid DNA from a cell. Curing of plasmid is done to determine whether a plasmid encode a trait or not. A trait is said to be plasmid –borne if plasmid encodes information about it. Curing of plasmid is being done using any of these: Novobiocin, Ethidium bromide (EtBr), Acriflavin, Acridine orange dye, Plumbagin, and Sodium Deodecyl Sulphate (SDS) Rahbar, *et al*., 2010; Bojary., 2012).

In this study Sodium Deodecyl Sulphate (SDS) was used as the curing agent. The procedure is described below.

ANTIBIOGRAM OF POST PLASMID CURING

Out of the eight isolates that were multidrug resistant that were subjected to plasmid curing number two was negative which was *Staphylococcus aureus* seven were positive for plasmids and the number five has multiple plasmids of 48.5kbp, 8kbp and 1.5kbp. 5 (63%) of the isolates were susceptible to antibiotics that they were formally resistant to. These include number 1 *Escherichia coli number 2 Staphyloccus aureus* number 4 *Proteus mirabilis, number 6 Citrobacter freundii*and number 7 *Klebsiella pneumoniae*. And 3 (37%) of the isolates were still resistant to the antibiotics like Cefuroxime, Erythromycin, and Cefotaxime.

Those that were now susceptible might be due to the fact that the plasmids borne multidrug resistant gene had been denatured or removed by the sodium deodecyl sulphate used as the curing agent. And the gene were said to be plasmid mediated and those that were not cure which are still resistance have their gene for resistant resident in extra chromosome. And these included number 3 Pseudomonas aeruginosa, number 5 *Proteus vulgaris,* number 8 *Enterobacter aerogenes*.

RESULTS

The results obtained are shown in tables 1-3 and plate 1

Table 1: Enumerates the different characteristics of the positive blood culture of neonates; the descriptions that include male- female weight less than 2.5 kg and those above 2.5 kg; inborn and outborn babies, pretem, term and post term babies and as well as age distributions.

Table 2: This shows the frequency of the isolates with *Klebsiella pneumoniae* being the most common pathogens 16 (27%) and closely followed by *Staphylococcus aureus* 14 (23%) while the least were Proteus vulgaris and *Pseudomonas aeruginosa* 3% each.

Table 3: shows the predominance of Gram negative organisms which is 46 (77%) over Gram positive organisms which is 14 (23%) of the isolates.

Plate 1: shows the results of the plasmid DNA profiling analyzed in 0.8% agarose gel Electrophoresis. Seven were positive for plasmid gene, out of which one was positive for triple plasmids genes while one was negative for plasmids gene.

Table 1: Characteristic of neonates with positive blood culture

Description	Study population n=300	Positive Blood culture n=60	Study population %
Male	180	38	21.11
Female	120	22	18.33
<2.5	86	18	20.90
>2.5	214	42	19.63
Outborn	120	20	16.67
Inborn	180	40	22.22
Preterm	78	36	46.15
Term	210	18	8.57
Post term	12	6	50.00
<72 hours	208	36	17.31
>72 hours	92	24	26.09

Trir 86=able 2: Frequency distributions and percentage of isolates from blood culture

Organisms	*Escherichia coli*	*Klebsiella pneumonia*	*Citrobacterfreundii*	*Proteus mirabilis*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Enterobacteraroges nes*
Number	10	16	4	4	2	2	14	8
Percentage	16.67	26.67	6.67	6.67	3.33	3.33	23.33	3.33

Table 3: Predominance of Gram negative bacterial over Gram positive in neonatal blood culture

Organisms	Number	Percentage (%)
Gram negative	46	76.67
Gram positive	14	23.33
Total	**60**	**100**

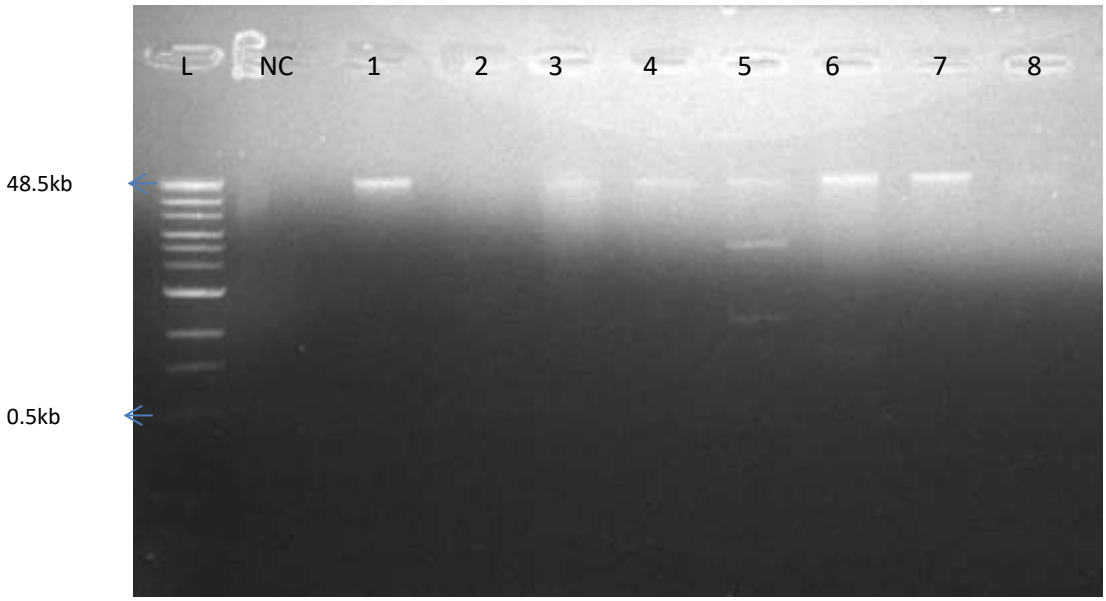

Plate 1: Plasmid profile of multiple drug resistance bacterial isolates analyzed with 0.8% agarose gel electrophoresis stained with ethidium bromide. L is 0.5kb - 48.5kb DNA ladder (molecular marker). Samples 1, 3, 4, 6, 7 and 8 are positive for plasmid genes with band at 48.5kb, sample 5 is positive for plasmid genes with triple bands at 48.5, 8 and 1.5kbwhile sample 2 is negative for plasmid genes. NC is a non plasmid DNA template control.

Key
1 = *Escherichia coli*
2= *Staphylococcus aureus.*
3= *Pseudomonas sp.*
4= *Proteus mirabilis*
5= *Proteus vulgaris*
6= *Citrobacter sp.*
7= *Klebsiella sp.*
8= *Enterobacter sp.*

Discussion

It was found from our study that Gram negative bacteria were the major cause of neonatal sepsis which account for 46 (77%) of the isolates and was in agreement with the study of Omoregie *et al.,* (2013). *Klebsiella pneumoniae* 14 (27%) was found to be the most common pathogen implicatedShresthaet *al.,*2010. This was closely followed by Gram positive organisms *Staphylococcus aureus* (23%) of the total isolates and it was followed by *Escherichia coli* (16%) while the least were *Proteus vulgaris, Pseudomonas aeruginosa*, and *Enterobacter aerogens* which were 3% each.

This can be attributed to the fact that neonate acquired these bacteria from the vagina of the mother and the instrument used in taking delivery and as well as the environment where the neonates were delivered

In our study, Ofloxacin which belong to the group of Quinlolones was observed to be the most potent antibiotic against both Gram negative and Gram positive organisms. This was followed by Amikacin and Gentamicin in the family of Aminoglycoside group, and followed by Augmentin (Amoxicillin and Clavulanate Acid) the Penicillin group, again followed by Cefotaxime the Cephalosporin group while Erythromycin a Macrolides and Cefuroxime a Cephalosporin showed very low activity. Change in Antibiotic susceptibility pattern could be explained by the fact that bacteria tend to develop resistance to those antibiotics that they are frequently exposed to, thus resulting of gene mutation that could take place in the bacteria and by acquiring resistance plasmid from other bacteria or from the environment. Low dosage could also lead to antibiotic resistance as well. It was also reported by researchers (Alsultanet *al,* 2013) and(Alanaziet *al,* 2018) that Extended Spectrum Beta-Lactamase (ESBL) producing *Esherrichia coli* are highly resistantantibiotics.

An antibiogram post plasmid curing of multidrug resistance showed great improvement in some of the clinical isolates that were formally resistant to the antibiotics were now susceptible. These might be due to the fact that the plasmids borne multidrug resistant gene had been denatured by the Sodium DeodecylSulphate used as the curing agent, and those that were not cure which are still resistant have their gene for resistant in extra chromosome.(Zamanet *al* 2010; (Inuzukaet *al,*2018) had recommended Sodium Deoecyl Sulphate as curing agent. While Aja*, et al* reported that the effectiveness of the curing agentsvaried from one study to others. According to some literature Zaman *et al,*2010, Alsultan *et al,*2013, and Elias *et al* 2013 reported 50% of the clinical isolates with high molecular weight of 23kb -26kb are multi-drug resistant because they possessed plasmids (Zaman *et al* 2010, Alsultan *et al* 2013, Elias *et al* 2013).

The result of the plasmid analysis that was carried out showed that there were detectable plasmid DNA genes in sample number 1, 3, 4, 6, 7, and 8 while sample number 5 was positive for plasmid genes with triple bands at 48.5, 8, and 1.5 kb. It is evident that the plasmid DNA gene isolated in the 0.8% Agarose gel electrophoresis was the bacteria content that helps in the multidrug resistance and this is in agreement with some research report (Ehiaghe *et al*., 2013). It was also reported that multiple plasmids with varying sizes in plasmid DNA that tend to be multi-drugs resistant. This is due to the spread of plasmids which could influence the susceptibility of the clinical isolates and also other mobile carriers of resistant genes that they possessed.

CONCLUSION AND RECOMMENDATION

In this study we were able to establish that those isolates that were multi-drug resistance harbored one or two or more plasmids which made then to be multi-drug resistant. And it was also observed that the curing agent has the ability to reduce the multi-drug resistance factor to the minimum. Hence curing is very useful in reducing multidrug resistance of pathogens thereby rendering those that were previously resistance, to become susceptible.

Conflict of interest Statement: The authors declare no conflict of interest.

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