

<https://dx.doi.org/10.4314/jnoa.v25i1.8>

## Isolation of plasmid genes in eye swabs of babies delivered through spontaneous vaginal deliveries and caesarian section.

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### Abstract

**Purpose:** A number of eye disorders arising from bacterial infection of an altered normal flora can affect new born babies. The study aimed to investigate the presence of plasmid genes in eye swabs of babies delivered through spontaneous vaginal deliveries (SVD) and caesarean Section (CS).

**Materials and Methods:** This was a three-month prospective cross-sectional experimental study. Eye swabs of new born babies delivered in the maternity ward of Aminu Kano Teaching Hospital were taken. Samples were collected within 30 minutes of delivery from 82 neonates (50 SVD and 32 CS). They were evacuated to the microbiology laboratory for culturing, characterisation and sensitivity. Isolates that were resistant to 3 or more antibiotics were tested for the presence of plasmid genes. Those that contain plasmid genes were subjected to curing using standard procedures..

**Results:** The result showed that 16(32%) of *E. coli*, *P. aeruginosa*, 14(28%), and *S. aureus*, 11(22%) was isolated in the SVD group samples as compared with 10(31.3%) of *S. aureus*, *E. coli*, 9(28.1%), and *P. aeruginosa*, 8(25.0%) among the CS group samples. There were 47(77.0%) SVD and 14(23.0%) CS samples that were resistant to three (3) or more antibiotics but this difference was not statistically significant ( $p=0.157$ ). Only 9(14.7%) isolates carried plasmid genes.

**Conclusion:** Plasmids genes were responsible for the resistance and promethazine is a good anti-plasmid agent. We recommend further research on the medical importance of anti – plasmid effect of promethazine working in synergism with other common antibiotic treatment to reduce treatment cost of plasmid-induced drug resistance.

**Keywords:** Spontaneous Vaginal Delivery, Caesarian Section, Antimicrobial resistance, Plasmid, Plasmid curing, Promethazine.

## Introduction

Neonatal eye infection occurs in babies exposed to infection during delivery, particularly during passage through an infected birth canal;<sup>1,4</sup> especially in Spontaneous Vaginal Delivery (SVD), that is without the use of tools like forceps or vacuum to bring out the baby. In-term babies, pre-mature rupture with a prolonged time before the onset of labour enhances the risk of neonatal eye infection because it usually leads to frequent vaginal examination (especially digital examination) which may contaminate the foetus exit parts with microbial agent<sup>5-11</sup>. In all pregnancies, premature rupture of membrane (PROM) may be seen in about 10.7%<sup>5,6</sup>.

Pre-term or low birth weight new born babies are at higher risk of infection, especially when there is incidence of PROM during labour<sup>10,11</sup>. Newborn eye infection may be due to gram positive bacteria or coagulase positive Staphylococcus<sup>12</sup>. Other bacterial pathogens that can inhabit neonate conjunctiva at birth include Streptococcus pyrogenes, Pseudomonas

aeruginosa, Viridians streptococcus<sup>12</sup>.

In Caesarean Section delivery (CS)<sup>13-16</sup>, babies do not come in contact with the natural birth canal. However, it has been reported<sup>16</sup> that growth yields of Staphylococcus aureus, Corynebacterium and Propioni bacterium acnea were higher in CS when compared to SVD. These may have been acquired primarily by the presence of bacteria in the surrounding air and also through the level of care given by the hospital personnel<sup>17,18</sup>.

The microorganisms in the maternal birth canal usually affects the flora in the early neonatal life of infants born through Spontaneous Vaginal Delivery (SVD)<sup>18,19</sup>. Thus, by contact with the vaginal secretions and the saprophyte and/or disease-causing bacteria present in the mother's birth canal, neonatal conjunctival bacterial community appears or neonatal conjunctivitis develops<sup>20</sup>.

Antibiotic resistance has been identified as one of the three most important threat to public health in the 21st century<sup>21</sup>. Multidrug –resistant

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(MDR) organisms caused infection are directly linked with high rate of premature death and they amount to a huge economic burden to many nations in form of incapacitation, long lasting illness leading to longer days spent in the hospital, the need for very costly medications and a financial burden to those affected, and it may cause a devastating repercussion on child's quality of life if not properly treated<sup>22,23,25</sup>. The development of bacteria with antibiotic resistance poses a challenge to the effective treatment of neonatal ocular infections. The result of this drug resistance is that antibiotics become impotent and infection becomes impossible to treat<sup>24</sup>. Some factors that may lead to antimicrobial resistance development include: Wrong use and over use of antimicrobials as the main driver, poor sanitation, inadequate infection prevention and control which encourages the spread of microbes some of which possess antimicrobial resistance ability, ignorance and absence of enforcement of legislation among others<sup>25</sup>.

Micro-organisms are capable of acquiring resistance to antimicrobials through several mechanisms such as: Enzymatic modification of drug, prevention of drug penetration into or accumulation of drug inside the bacterial cell, target over production or enzymatic by-pass, target mimicry and by altering the target enzyme

of the antimicrobial agent<sup>26</sup>. Apart from these, some micro-organisms have acquired more than one resistance mechanism, making them resistant to several antimicrobials' agents<sup>26</sup>. Plasmid genes have been found to be responsible for drug resistance and they can easily move between microorganisms through lateral or horizontal gene transfer (HGT), in which there is a partial exchange of genes in their biofilm state<sup>27,28</sup>. Plasmids are extra chromosomal, independently-replicating genetic materials found in cells that is different in every way from chromosomes (i.e., they are not a part of the cell's genetic make-up) and usually grants a specific attribute to a bacterial cell. These attributes include antibiotic resistance, reproduction, toxin production and many other features<sup>29</sup>

Plasmid curing of a bacterial cell is a means to remove plasmid gene in bacteria and find the antibiotic that can be used to kill the bacteria. It may happen on its own through cell division or by subjecting the cell to treatment with chemical or physical agent, thus leading to stopping of conjugal movement of antibiotics resistant plasmid, thereby giving rise to a reduction in the spread of drug resistant plasmid bearing cells which makes it a difficult task to find therapy to these infections<sup>30</sup>.

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The study was aimed at investigating the presence of plasmid genes in isolates from eye swabs of babies delivered through spontaneous vaginal delivery and caesarean section and also to find a possible cure.

### Materials and Methods:

This is a 3 months cross sectional experimental study carried out at the labour ward, maternity theatre of Aminu Kano Teaching Hospital (AKTH), Kano while the laboratory analysis was done at the department of Microbiology and at the centre for biotechnology research, Bayero University, Kano. The sample size was determined using the Cochrane method<sup>31</sup>, giving a total of 82 neonates, of which 50 were delivered through SVD and 32 through CS. Samples from one eye of the new born babies who met the inclusion criteria which include babies delivered at AKTH only, who had no ocular morbidity nor had received any ocular prophylaxis at birth and whose parents gave assent were collected within 30 minutes of birth, by gently rolling a sterile swab stick along their conjunctival membrane while wearing a sterile hand glove. The swab stick containers were numbered and labelled serially as collected for easy identification. Due to high environmental temperatures, all samples collected were stored in the refrigerator maintained at 4°C and evacuated in ice pack containers in batches within 24 hours to the microbiology laboratory which is about 10

kilometres away, for culturing, characterisation and sensitivity profiling. Isolates that were resistant to 3 or more antibiotics were analysed for the presence of plasmid genes. Those that contain plasmid genes were subjected to curing using standard procedures.

### Processing of samples

Swabs were anaerobically cultured in sterile Blood agar, MacConkey agar, Nutrient agar Mannitol salt agar and Muller-Hinton agar at 37°C for 24 hours. This is to give room for all possible organism in the specimen to grow. Isolates were identified culturally, morphologically and biochemically according to standard protocols<sup>32,33</sup>. Antibiotics susceptibilities of the isolates were determined using the disc-diffusion method. The result of morphological identification, characterization and sensitivity profiling of all the bacteria isolated were observed and recorded. Isolates from samples that were resistant to three or more antibiotics were then inoculated into nutrient broth contained in bijoux bottles and sent to the biotechnology laboratory for analysis for the presence of plasmid gene. Those isolates in which plasmid gene were extracted were subjected to curing using standard procedures.

### Plasmid Extraction from Isolates

Plasmid extraction from the bacteria samples was done using the manufacturer's protocol

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of plasmid mini-prep kit from Norgen Biotek Corporation<sup>®34</sup>. The solution was centrifuged at 14000rpm for 1 minute and the supernatant discarded. Lysis buffer (400µl) from the kit and proteinase K (15µl) were added to the pelleted bacteria sample, then vortexed and incubated. Absolute ethanol (400µl) was added to each solution before it was transferred into the spin column for centrifugation, followed by series of washing with different buffer solutions provided. Finally, 50µl of DNase and RNase free water was added to the spin column to elute the plasmid material into 1.5ml micro centrifuge tubes. The extracted products were subjected to gel electrophoresis using 1% agarose gel powdered. The gel was viewed using clever gel documentation system. Isolates that contain plasmids were then returned to the laboratory in nutrient agar and subjected to the curing processes.

### Plasmid Curing

This was done using the protocol used by Molnar et al, (2003)<sup>35</sup> with modification. 0.9ml of freshly prepared nutrient agar was added into bijou bottles and autoclaved for 15 minutes and allowed to cool. Using a sterile wire loop, the bacterial cells bearing plasmid gene were inoculated into the nutrient agar and incubated for 24hours at 37°C. From a concentration of 50mg/2ml promethazine hydrochloride ampoule, 0.1ml (equivalent to 2.5mg/ml) was pipetted into

the bijou bottles and incubated again at 37°C for 24 hours after bacterial growths were observed. They were then sent to the molecular laboratory to confirm the presence of plasmids or not. Thereafter, the samples were again returned to the laboratory and cultured anaerobically in blood agar at 37°C for 24hours and the plates observed for any bacterial yield.

### Ethical Approval

Informed consent was sought from parents and relevant authorities prior to the study. Ethical approval was obtained from the research ethics committee of Aminu Kano Teaching Hospital, with Ref. NHREC/21/08/2008/AKTH/EC/2786 and dated, 17th February, 2020. The study adhered to the tenets of the declaration of Helsinki.

### Results

Determination of the presence of plasmid genes in isolates from eye swabs from conjunctiva of a total of eighty-two eyes (n=82) of neonates comprising 50(61.0%) delivered through spontaneous vaginal delivery (SVD), and 32(39.0%) delivered through caesarian section was carried out in this study. The result of the identification of organisms isolated is presented in table 1. Four distinct bacteria were morphologically identified from all the samples.

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**Table 1: Bacterial isolates identified from the samples**

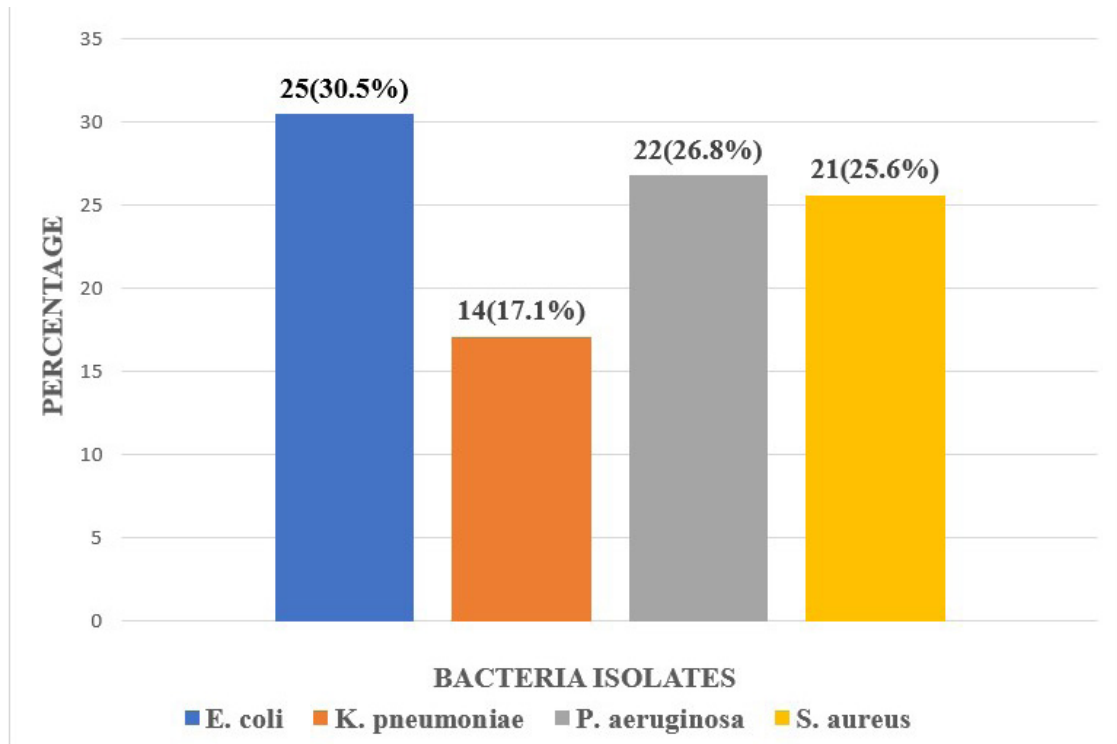
S/N	Colonial appearance after incubation	Morphological characteristics	Bacterial species
1	Colonies appeared as circular, low convex, entirely opaque, shiny and butyrous, ranging from creamy white to deep golden colour grown in nutrient agar plate after 24hrs incubation at 37oC.	They are uniformly gram-positive (+ve) cocci, arranged in irregular, grape-like clusters. They are also in pairs and some in singular cells	<i>Staphylococcus aureus</i>
2	They appeared as pinkish, metallic and golden, shiny colonies on eosin methylene blue agar plate after 24hrs incubation at 37oC	They are uniformly gram-negative (-ve) short and plump-rods cells in appearance	<i>Escherichia coli</i>
3	Light pink, viscid, mucoid and swarming colonies appears on McConkey agar plate after 24hrs incubation at 37oC	Short and fairly long bacilli. Produced a capsules and non-motile cells, stained uniformly gram-negative (-ve) bacilli	<i>Klebsiella pneumonia</i>
4	The colonies appear greyish in colour Grown aerobically at 37oC with opaque smooth transparent appearance on nutrient agar plate after 24hrs incubation	They are uniformly gram-negative (-ve) rod-shaped, non-sporing	<i>Pseudomonas aeruginosa</i>

Biochemical characterization tests were performed to confirm at specie level, the bacterial isolates. Four distinct bacterial species were isolated as shown in table 2.

**Table 2. Biochemical characterization test for confirmation of the bacteria isolated at specie level.**

S/N	Tests	Bacterial Isolates	Remarks
1	Catalase Test	<i>Staphylococcus aureus</i>	+
	Coagulase Test		+
	Mannitol Test		+
	Glucose Test		+
2	Indole Test	<i>Escherichia coli</i>	-
	Methyl red Test		-
	Voges Proskaver Test		-
	Citrate Utilization Test		-
3	Mannitol Test	<i>Klebsiella pneumonia</i>	+
	Indole Test		-
	Methyl red Test		-
	Voges Proskaver Test		+
	Citrate Utilization Test		+
4	Cytochrome Oxidase Test	<i>Pseudomonas aeruginosa</i>	+

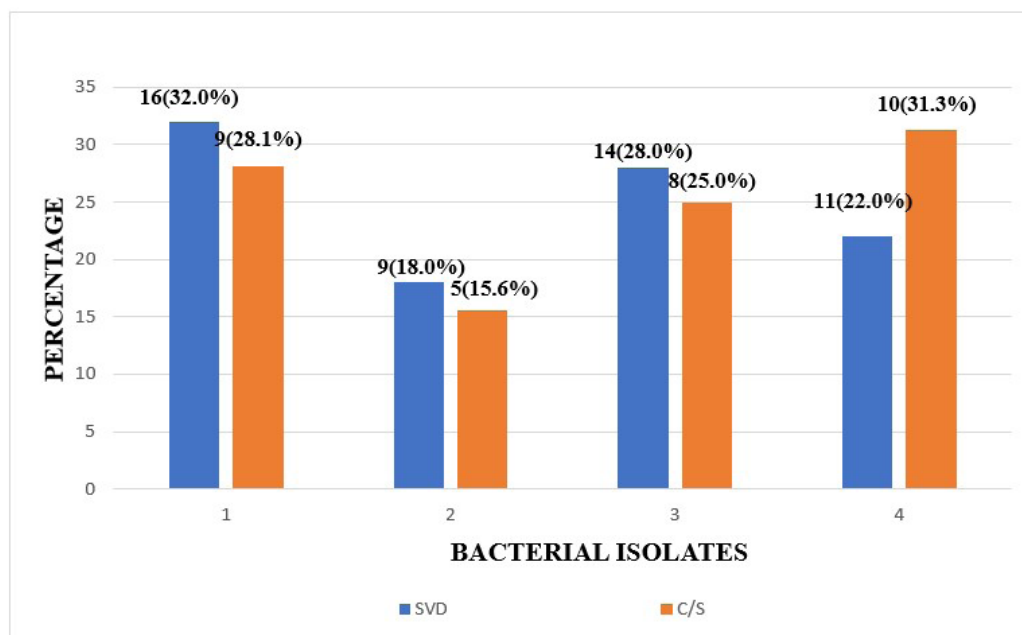
Sensitivity to antibiotics of isolates from all the samples showed that *E. coli* was the predominant organism with the highest sensitivity 25 (30.5%) of the samples while *K. pneumonia* was the least 14 (17.1%) of the samples as shown in Figure 1



Data presented in percentage and frequency.

**Figure 1. Distribution of bacterial growth yield from all the samples**

The Distribution of bacterial isolates from all the samples showed that *E. coli* was the predominant organism with the highest percentage 25 (30.5%) of prevalence among the samples while *K. pneumoniae* was the least 14 (17.1%) of the samples as shown in Figure 1.



Data presented in frequency and percentage. Key: 1= *E. coli*, 2 = *K. pneumoniae*, 3 = *P. aeruginosa*, 4 = *S. aureus*.

**Figure 2: Bacterial Growth Yield by Mode of Delivery.**

Figure 2 showed that *E. coli* and *S. aureus* were mostly isolated in babies delivered through SVD and C/S respectively, while *K. pneumoniae* was the least isolated in both mode of deliveries. However, comparing the isolates among the SVD, *P. aeruginosa* 14 (28.0%) was the second most isolated bacteria followed by *S. aureus* 11 (22.0%). While among the C/S, *E. coli* was the most second isolated organisms followed by *P. aeruginosa* 8 (25.0%).

**Table 3: Distribution of Isolates Resistance to various antibiotics between SVD and CS groups.**

Profile	SVD n (%)	C/S n (%)	Total n (%)
Resistant to less than 3 antibiotics	3(14.3)	18(85.7)	21(100)
Resistant to 3 or more antibiotics	47(77.0)	14(23.0)	61(100)

The frequency and percentage of isolates resistance to various antibiotics from samples collected from babies born through SVD showed that 47 (77.0%) samples were resistant to three (3) or more antibiotics, while 14 (23.0%) were observed in CS group as indicated in table (3).

**Table 4: Relationship of Isolates Resistance to various antibiotics between SVD and C/S groups.**

Profile	SVD n (%)	C/S n (%)	Total n (%)	chi-square	p-value
Resistant to less than 3 antibiotics	3(14.3)	18(85.7)	21(100)	2.000	0.157
Resistant to 3 or more antibiotics	47(77.0)	14(23.0)	61(100)		

The frequency and percentage of isolates resistance to various antibiotics from samples collected -from babies born through SVD showed that 47 (77%) samples were resistant to three (3) or more antibiotics, while 14 (23%) were observed C/S group as indicated in table (3). Pearson chi square showed that there was no relationship between the resistance of isolates from SVD and C/S samples to antibiotics and were statistically insignificant.

**Table 5: Outcome of plasmid extraction processes from the Isolates in both the SVD and CS groups.**

Isolates	SVD n (%)	C/S n (%)	Total n (%)
E. coli	1(11.11)	1(11.11)	2(22.22)
S. aureus	3(33.33)	2(22.22)	5(55.56)
P. aeruginosa	-(0.00)	1(11.11)	1(11.11)
K. pneumonia	1(11.11)	-(0.00)	1(11.11)
<b>Total</b>	5(55.6)	4(44.4)	9(100)

Of the 61 samples that were resistant to 3 or more antibiotics, plasmid genes were extracted from 9(14.75%) isolates with *Staphylococcus aureus* bearing most of it in 5 (55.6%) across both mode of deliveries. Plasmids were extracted more from the SVD group in 5(55.6%) while 4(44.4%) was extracted from the C/S group as shown in table 4.



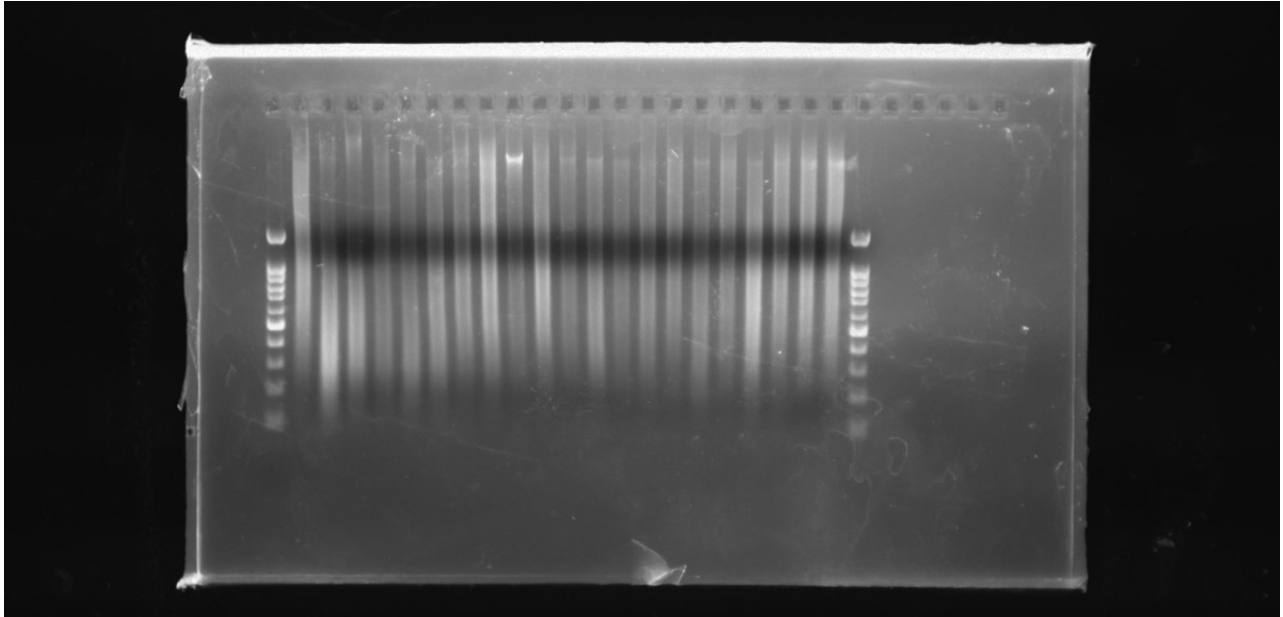


Figure 3: **Showing Plasmid isolation process.** The image is a representative of the process with the comb containing 21 samples. At the end of extraction process, 9 bacterial cells had plasmids genes in them which were seen as a white band at the top of the well as captured after electrophoresis through the Gel documentation system.

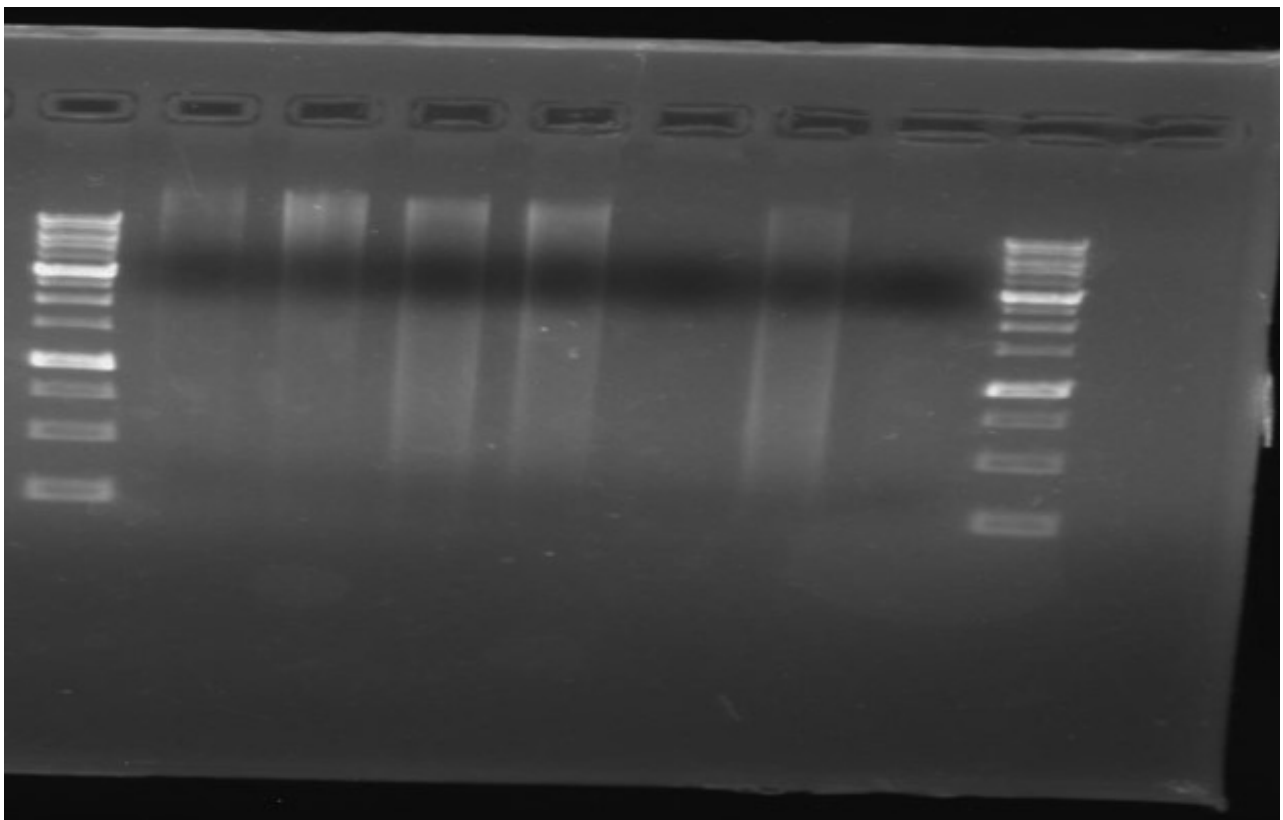


Figure 4: **Showing Plasmids after being cured.** The white bands that signifies the presence of plasmids are no more, indicating that the plasmid genes have been deleted (cured) from the bacterial cells.

## Discussion

The study investigated the presence of plasmid genes in isolates from swabs of eye lashes of babies delivered through Spontaneous Vaginal delivery and Caesarian Section. It was observed that all the 82 samples were culture positive and four distinct and similar bacterial growth yield was isolated across both mode of deliveries.

The microbial flora of babies delivered through SVD and those of C/S does not differ as both mode of deliveries yielded same growths. However, *E. coli* was mostly isolated from the SVD group, while *S. aureus* was the most common isolate among samples in the CS group. Studies have shown that microbial community of the mother's vagina usually affects conjunctival flora in the earliest stage of life in neonates born through SVD<sup>18,19</sup>. This is because the baby comes into contact with vaginal secretions and saprophyte or pathogen bacteria present in the birth canal, leading to the emergence of newborn conjunctival flora which may lead to development of neonatal conjunctivitis. This may explain the higher growth yielded among these babies.

On the other hand, in babies born through CS, the new born do not come into contact with the birth canal. A study of babies born through CS showed that *S. aureus* was the most isolated organism perhaps due to their non-contact with the vagina. Colonization of the ocular

conjunctiva in new born delivered through CS usually occurs within the first day of life<sup>16</sup>. *S. aureus* is a gram positive, non-motile, round shaped bacteria and is the most common cause of infection after injury or surgery. It is found in human skin, nose, groin, armpit contaminated water and it is transmitted through close contact with contaminated surfaces or sharing contaminated items. *S. aureus* is found in indoor and farm environments as a component to airborne dust, but the highest concentration is found in high hand touch areas such as door knobs, suggesting that human contacts in crowded areas such as in hospitals/academic institutions may play crucial role in *S. aureus* transmission via inanimate objects<sup>17</sup>. This agrees with the findings in this study. Since the neonate born through CS does not contact the maternal birth canal, it means that these florae may be principally acquired through the presence of bacteria in the surrounding air as well as level of care given by the hospital personnel<sup>17</sup>.

Another origin of neonatal infection is often through maternal gastro intestinal and genito-urinary tracts. This is because many of the mother's infections with these microbes do not disturb her<sup>4</sup>. *E. coli* is a rod-shaped bacterium of the family, Enterobacteriaceae. It is found in the environment, contaminated food and water, and lives harmlessly in the gastro-intestinal tract of healthy people. A particular strain of this organism is known to be pathogenic if it

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gets into the GIT by ingestion and later, passed out through the stool. Those mostly at risk of infection are newborn babies and pregnant women due to their low immunity. And because the mother's urethra lies close to the anus, the bacteria get easier access to the bladder where majority of urinary tract infections occur. This predisposes the mother to develop genitourinary tract infection, which may be a source of infection in the eye of babies who were vaginally delivered<sup>4</sup>. This may also explain why *E. coli* was the most isolated organism from the test samples in this study

Distribution of isolates resistance to various antibiotics between both modes of delivery showed that from the 82 samples, 61 isolates were resistant to 3 or more antibiotics. Pearson chi square showed that there was no relationship between the resistance of isolates from SVD and C/S samples to antibiotics and were statistically insignificant (Table 4). The choice of three as a baseline was arbitrary, but we considered any isolate resistant to a minimum of three or more antibiotics as an isolate of interest, bearing in mind the consequences of antimicrobial resistance (AMR) and its importance to the health economics of a nation. This is because it affects output of patients and those who care for them especially through longer stay in the hospital and the need for expensive and intensive care. Therefore, in the absence of powerful antimicrobials, the expected outcome

of contemporary medicine in treating infections especially during major surgery and cancer drug therapy would be at highest risk<sup>25</sup>.

A further look at the distribution of isolates resistance to antibiotics showed that resistance to three or more antibiotics was greater in the SVD group when compared with the C/S group. This agrees with findings of Mustafa et al 2018.<sup>16</sup> This may be due to reasons mentioned above as proffered by those authors. Also, exposure of pathogens to anti-microbial agents is the main reason why resistance occurs. Genes responsible for resistance can mutate and then be transmitted asexually or by horizontal gene transfer (HGT), even in their biofilm state to the next microbial generation until they become the dominant population<sup>27</sup>. HGT is one of several mechanisms<sup>24</sup> through which there could be exchange of resistant genes among microorganisms. There are five major classes of plasmids<sup>36</sup> and they are also very useful especially in areas of genetic engineering and biotechnology<sup>37</sup>, however, they have been found to be efficiently responsible for conferring drug resistance capacity on bacteria<sup>27</sup> and are easily transferred by HGT. Since the disparity in population size of both groups is skewed in favour of the SVD group, together with events that may happen during the labour hours of the mother, it is possible that isolates from the SVD group may show more resistance compared to the C/S group.

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The outcome of the plasmid extraction showed that 9 bacterial cells have plasmid bearing genes. Plasmids were found mostly in *Staphylococcus aureus* and in isolates originating from the SVD babies. The reason for this is not known, however, antimicrobial resistance genes (ARGs) are very often found in plasmids. The presence of plasmid in a bacterial cell encodes various properties to it like resistance to antibiotics and heavy metals, degradation of hydro carbons, synthesis of bacteriocins etc<sup>38</sup>.

New and interesting techniques to combat AMR is emerging and plasmid curing can lower ARG prevalence and therefore, make bacteria sensitive to antibiotics. There are many compounds that have exhibited plasmid curing ability and they vary greatly in their effectiveness and depends on the bacterial specie, type of plasmid and growth conditions<sup>38</sup>. Promethazine (in the family of phenothiazine), a widely and safely used antihistamine and antiemetic drug in everyday medical practice and which has no remarkable effect on the central nervous system<sup>39</sup> have been recognized as a powerful anti-plasmid agent in cultures that have more than one bacterial species<sup>35</sup>.

The mechanism of action of promethazine in plasmid curing is by the selective stopping of plasmid replication over that of plasmid carrying bacterium at three different levels or steps<sup>39</sup>. Promethazine was also taught to potentiate the antimicrobial effect of different antibacterial

agents by increasing cell membrane permeability, thereby eliminating the resistant plasmid gene in the bacterial cell<sup>39</sup>.

In a study by Bucknor et al, 2018,<sup>38</sup> the effect of promethazine on a culture of bacteria of diverse species showed that plasmid curing was obtained at different concentrations but greater effect was observed at higher temperatures under the same culturing conditions. The authors concluded that multi-species co-cultures lowered promethazine concentration needed for curing and that cure was more effective at elevated temperatures.

In this study, 2.5mg/ml in 0.1ml of promethazine was used to obtain plasmid elimination at 37oC and 24hours incubation. The reason for obtaining cure at this condition may be due to higher concentration of the promethazine which may be above the minimal inhibitory concentration needed for plasmid elimination and also the fact this is not a mixed bacterial culture. In comparison with a study by Molnar et al, 2003,<sup>35</sup> it implies that higher concentration of promethazine will affect plasmid curing in single bacterial cultures but at a lower temperature.

### Study limitations.

The scope of this study did not cover the period of ante natal activities of the mother of the babies. This would have helped to know the events that occurred prior to labour and delivery and then understand if these may have affected study outcomes. Again, the biotechnology

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laboratory does not have the ladder which could read the molecular weight of the plasmid DNAs identified. This is due to high cost and also scarcity. Knowing their molecular weights would have aided to identify the type of plasmid genes present in the isolates. We could not determine whether the plasmids were acquired or innate to the isolates due to paucity of funds. This would have helped in advising the hospital to seek ways to improve the hygiene of the maternity and labour wards

## Conclusion

Neonates born through SVD have most antibacterial resistance when compared to those delivered through CS. This means that they are more susceptible neonatal conjunctivitis probably due to the presence of multiple-drug resistant bacteria. In view of the economic importance of anti-bacterial resistance, there is an urgent need to develop novel drugs with antibiotic capacity against plasmid induced multi-drug resistance bacteria which causes grave infections that are highly challenging to treatment and sometimes untreatable. Research have established that promethazine, when used in synergy with regular and available anti-bacterial agents eliminates the effect of plasmid induced drug resistance. This will reduce treatment cost certainly. Therefore, we recommend further research in the medical importance of anti – plasmid effect of promethazine working in synergism with other common antibiotic treatment. This will open a new vista in pharmaceutical drug formulation against multi drug resistant bacterial infections. The consequence of such breakthrough in innovative drug will be that old and cheap antibiotics which will provide therapeutic solution to plasmid induced antibiotic resistant infections will be available.

## Financial Support and Sponsorship

Nil.

## Conflicts of Interest

There is no conflict of interest.

[ How to cite this article: Muoneke H.C., Ebisike P.I., Ajayi O.B., Hassan S., Habib S.G., Atuanya G., Okpo E., Lekan P.S., & Umar D.S. (2023) Isolation of plasmid genes in eye swabs of babies delivered through spontaneous vaginal deliveries and caesarian section. Journal of the Nigerian Optometric Association.2023;25(1):54-66 ]