Original Research Article

Levels of Biofilm Expression in *Klebsiella pneumoniae* strains exposed to Herbal Drugs

Background: There is continuous rise in antimicrobial resistance globally and factors responsible for this occurrence especially in developing countries are yet to be properly elucidated. Due the financial implications of antimicrobials individuals in developing countries such as Nigeria resort to the consumption of herbal drugs to treat infection.

Aims: To investigate the levels of biofilm expressed in *Klebsiella pneumoniae* pre-treated with herbal drugs.

Methodology: Biofilm assay was performed by using 24-well polystyrene plates which mimic the surface for bacterial attachment. Control and clinical strains were pre-exposed to different concentrations of herbal solutions (Beta cleanser [Bet], Goko alcoholic bitters [Gab], Goko bitters [Gob], Danko solution [Dan], and Ruzu bitters [Ruz]) (100, 50, 25, 12.5, and 6.25%) in 24-well plate and incubated overnight at 37°C. Cell-to-cell surface attachment of *K. pneumoniae* was recorded by obtaining a photograph of the inoculum in the 24-well plate. Crystal violet method was further used to quantify the level of biofilm attached to the surface of the 24-well plate. Results were anaylsed using student t-test with Graph pad prism 5.

Results: Cell-to-cell biofilm formation was seen in different drugs used but higher in Bet and Gob. Bet (25%) and Ruz (Ruzu bitter 50%) showed significant level of attached biofilm formed compared to untreated control. This results show that Bet, Gob and Ruz has the ability to induce biofilm in *K. pneumoniae*.

Conclusion: Herbal drugs could predispose *K. pneumoniae* to enhance its production of biofilm.

Keywords: Biofilm, Klebsiella pneumoniae, herbal drug, antimicrobial resistance

1. INTRODUCTION

Since the introduction of antimicrobial agents there have been several observations of the development of antimicrobial resistance in many species of bacteria. The first ‘miracle’ antibiotics discovered was Penicillin [1]. Resistance to Penicillin was later known to have been caused by Penicillinase, a member of β-lactamases that cleaves the benzylpenicillin. In less than 20 years of the introduction of Penicillin, a rapid increase in the production of penicillinase was observed. This observation was noted for tetracycline, penicillin and macrolide at the end of 1950s. This led to the generation of different strains of microbes, resulting in difficulty in management of infections.

Antimicrobial resistance is a serious health concern that impedes the management and prevention of infections. Different cases of antimicrobial resistance have been seen around the world [2]. Some of these cases of antimicrobial resistance include the emergence of resistant strains of tuberculosis have been discovered with 4.5 million recent cases of antimicrobial resistance in tuberculosis seen globally in 2012. Other cases of resistance have been observed in other bacteria pathogens such as *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*. *E. coli* resistance has now been seen in fluoroquinolone, a widely used antibiotic for the treatment of urinary tract infections. Some isolates of *S. aureus* have shown resistance to first-line drugs. Resistance in *K. pneumoniae* to carbapenem, a last resort antibiotic, is now in all parts of the globe [2].

A number of mechanisms for antibiotics resistance and spread have been discovered. The horizontal gene pool consisting of the mobile genetic elements is responsible for the lateral transfer of genes. This can occur either within individual species or among different species. Multidrug resistance mechanisms occur naturally via erroneously replication or transfer of resistant traits [3]. The force driving this process is the selective force of antimicrobial utilization. This is very notable in hospitals environment where clear correlation between antimicrobial use and development of resistance can be seen [4], [5], and [6].

The pathogenesis and outcome of *K. pneumoniae* infection depends on the virulence factors it produces in the course of the infection. An important virulence factor in this bacterium is the ability to produces extracellular polysaccharides called biofilm. Bacteria form biofilm in order to successfully invade and damage the host tissue. Biofilms are surface-attached extracellular polysaccharide matrix. It could lead to life-threatening bacteremia when formed on medical devices such as catheters [7]. Biofilms pose serious challenges to drug treatment by resisting antimicrobial actions at concentrations of up to a thousand folds that could easily eliminate free living or planktonic cells. Factors enhancing biofilm-mediated resistance characteristic include; reduction in the proliferation rate of biofilm [8], inefficient sequestering off antimicrobial agent within biofilm matrix [9] and presence of ''persister'' cells.

The aim of the current study was to examine the hypothesis that exposure of *K. pneumoniae* to herbal treatments could increase the production of biofilm. The results obtained were compared to a control conditions (untreated conditions). The data from the biofilm assay demonstrates that pre-exposure of *K. pneumoniae* strains to some herbal drugs not only results in surface biofilm but also increases *K. pneumoniae* biofilm attachment to polystyrene plate.

2. MATERIALS AND METHODS

2.1 Collection of Drugs

Locally-made drugs used in this study are Beta cleanser [Bet], Goko alcoholic bitters [Gab], Goko bitters [Gob], Danko solution [Dan], and Ruzu bitters [Ruz]. They were purchased from Mile 3 market, Port Harcourt, Rivers State, Nigeria.

2.2 Determination of the concentrations of the herbal drugs

The concentrations of the herbal antimicrobial solutions were determined by evaporating 1 ml of the different solutions to dryness in test tubes. The differences in the weight of the test tubes after dryness were determined. The different weights obtained were: Golden seed (0.5 g/ml), Ruzu bitters (0.29 g/ml), Beta Cleanser (0.09 g/ml), Goko Cleanser Herbal mixture (0.09 g/ml).

2.3 Collection of Organisms

The laboratory strain also known as control strain of *K. pneumoniae* ATCC 13883 was purchased from Sigma United Kingdom while the clinical strain was obtained from Lahor Research Laboratory, Benin, Edo State, Nigeria.

2.4 Media Preparations

2.4.1 Tryptone Soya Agar (TSA) and Tryptone Soya Broth (TSB)

The microbial media used were TSA and TSB. These were prepared according to the manufacturer's instructions and autoclaved for 15 minutes at 121°C. TSA was aseptically poured into sterile Petri dishes and TSB was stored in storage bottles for subsequent use.

2.5 Biofilm attachment assays

The biofilm assay used in this study is modified from the method used by Lyte et al. [11]. *K. pneumoniae* strains were grown in TSB overnight to log phase (Optical Density 0.5) were diluted to 1:100 in TSB supplemented with 100%, 50%, 25%, 12.5% and 6.25% of the various original concentrations of locally-made drugs [Bet, Gab, Gob, and Ruz] stated in section 2.2. A negative control was performed alongside. The cultures (200 µL) were transferred into a 24-well polystyrene microtiter plate (in triplicate wells). Wells containing sterile growth medium were carried out to check for contamination. The plates were incubated at $37^{o}C$ for 24 and 48 hrs and photograph of the surface biofilm were taken. The media and loosely adhered bacteria were removed by vigorously tapping the plate on a tray. Wells were re-washed three times with normal saline to get rid of any remaining nonadherent bacterial cells and media. Plates were air-dried at about 45°C for 1 hr. Bacteria wells were stained with 1000 µL of 2% crystal violet stain for 15 minutes at room temperature. After stain was removed, plates were washed twice in normal saline and plates were dried overnight. Plates were incubated in 1000 µL of 95% ethanol for 10 minutes to solubilise the crystal violet stains. The attachment of bacterial was quantified by measuring the absorbance of the crystal violet at 595 nm. The experiment was performed in triplicate on at least three separate occasions. Data were analysed on Graph Pad Prism 5.0.

3. RESULTS

3.1 Cell-to-cell attachment

K. pneumoniae showed a surface biofilm formation in Gob and Bet when viewed from the surface (Figure 1). No surface biofilm were seen in Gab and Ruz. The two highest concentrations of both drugs two did not show any level of biofilm induction. The clinical strain showed a more intense level of cell-to-cell aggregation compared to the control.

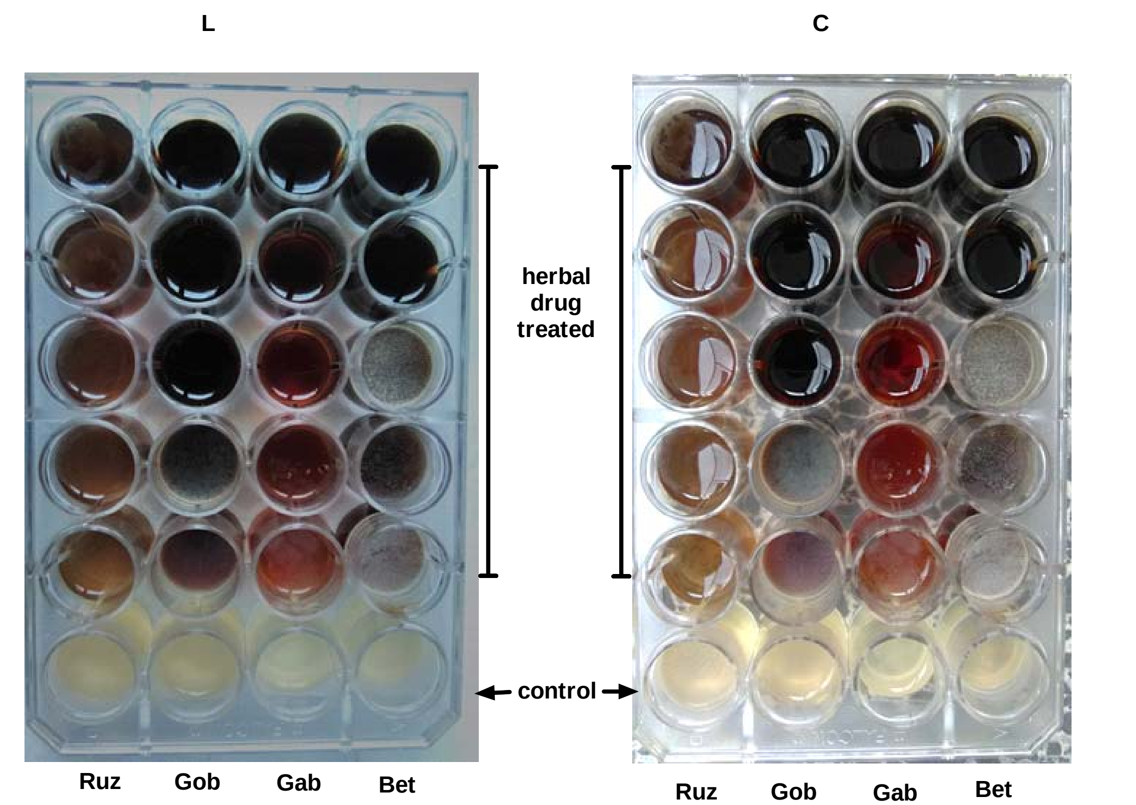
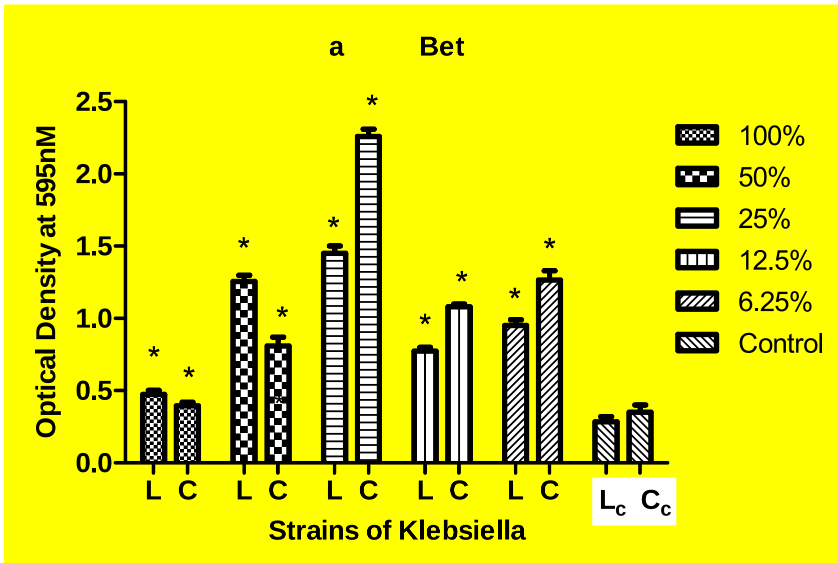
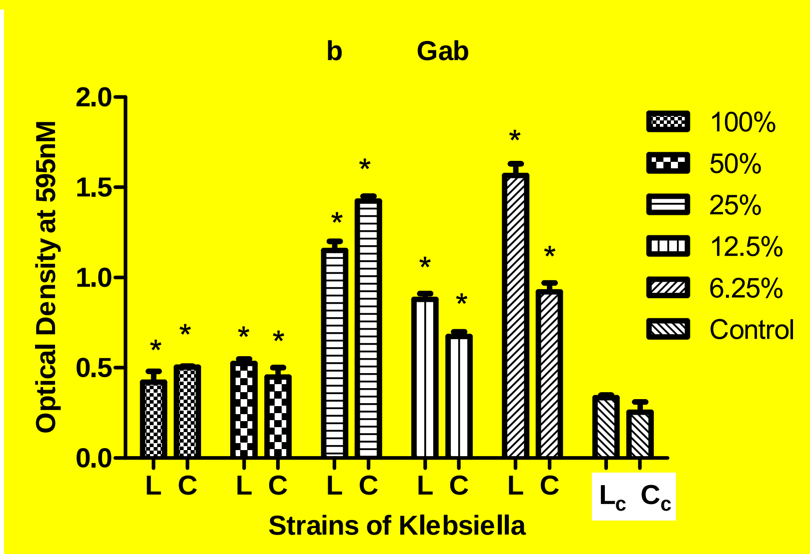


Figure 1. Surface biofilm formation in *K. pneumoniae* exposed to some herbal solutions. *Biofilm levels were analysed after 24 hrs of exposure to herbal preparations using spectrophotometer at 595 Nm. Beta cleanser [Bet], Goko alcoholic bitters [Gab], Goko bitters [Gob], Danko solution [Dan], and Ruzu bitters [Ruz], L: Laboratory strain, C: Clinical strain.*

3.2 Biofilm analysis with crystal violet assay

Figure 3.2 shows the level of biofilm produced in *Klebsiella pneumoniae* exposed and unexposed. In order to investigate the ability of *K. pneumoniae* to attach to surface of medical devices a modified method of crystal violet biofilm assay was used. The biofilm was detected as optical density measured at 595 Nm. In the experiment, all drugs showed higher levels of biofilm induction than the control condition (unexposed). There were similarities in the pattern of biofilm adherence to the polystyrene surface in the different drugs used (Figure 3.2a-d). The unexposed isolates are represented as L_C and C_C. A common trend observed in the experiment is that higher concentrations of the locally-made herbal preparations exhibited reduced level of biofilm production. The lower concentrations of the drug used showed a higher level of biofilm induction. The highest level of biofilm induction is observed in Bet (OD= 2.3), followed by Ruz (OD= 2.0), then Gab (OD= 1.5) and Gob (OD= 1.3). Figure 3.2a and b showed similar pattern of biofilm production: the 25% concentration showed much higher levels of optical densities. Bet (25%) and Ruz (50%) showed significant level of biofilm formed compared to untreated control.





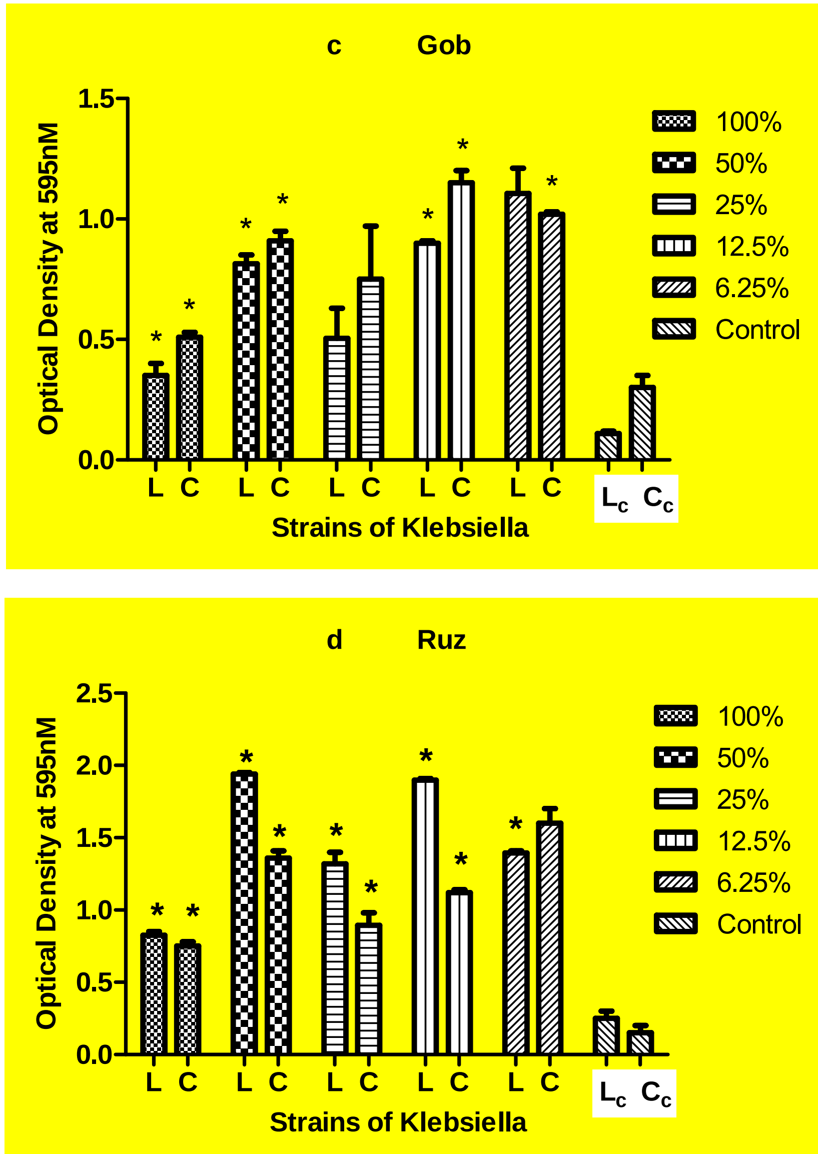


Figure 3.2. Levels of expression of Biofilm in *K. pneumoniae*. *Levels of biofilm formed were measured after 24 hrs incubation with and without herbal drugs at 595 nM. Data plotted above are mean ± standard deviation of three independent experiments performed in triplicates. * Level of significance compared to control not exposed to herbal drugs (L_c and C_c) using $p<0.05$). Beta cleanser [Bet], Goko alcoholic bitters [Gab], Goko bitters [Gob], Danko solution [Dan], and Ruzu bitters [Ruz], L: Laboratory strain, C: Clinical strain.*

4. DISCUSSION

There are two ways biofilm can be formed in bacteria; cell-to-cell aggregation and attachment to surface [10]. The potential of bacteria to resist antibiotics and form biofilm on medical devices is becoming high in hospital-acquired infections [11]. This investigation analysed the level of this virulence factor in *K. pneumoniae* exposed to some common herbal preparations used in Nigeria. The data on the drug resistance mechanism induction by herbal drugs furthers our understanding and appreciation of the possible cause of drug resistance in Nigeria.

The processes in bacterial biofilm formation firstly begin by the initial attachment to a surface [11]. Findings from other investigations have shown that pathogenic bacteria recognise inotropic drugs and use them to grow and produce biofilm [12] and [11]. However, information is yet available as to whether these herbal drugs induce biofilm in *Klebsiella* spp in similar fashion. Hence, the aim of this project was to investigate biofilm levels in *K. pneumoniae* strains response to exposure to herbal drugs. In this investigation, it was shown that concentration of herbal drugs within the range consumed could markedly increase biofilm levels of *K. pneumoniae* responsible for its ability to persist in the host.

Antimicrobial resistance is a growing problem in controlling infection and biofilm formation by *K. pneumoniae* is an aspect of *K. pneumoniae* pathogenicity that enhances its ability to colonise host. We demonstrated that herbal drugs most commonly consumed by sick patients (Bet, Gab, Gob and Ruz) all markedly increased *K. pneumoniae* biofilm formation on polysterene surfaces. This is a crucial discovery as bacterial ability to colonise surfaces such as catheters and other hospital plastic devices is a reason thought to influence patients to acquire pneumonia and other blood related infections [13, 14, 15].

Biofilm analysis of herbal drugs induction of biofilm observed in *K. pneumoniae* showed a minimum of two fold increase compared to control (Figure 3.2a) and a maximum of 8-fold increase (Figure 3.2d). A similar study by Freestone et al. [12] demonstrated that *Pseudomonas aeruginosa* a close organism also responsible for pneumonia-associated infection showed increase in biofilm level using crystal violet method. Their study showed a minimum of 1.5-fold increase and maximum of 2-fold induction caused by stress factor such as catecholamine. This is similar to the fold increase observed by Freestone et al [12] using catecholamines as a biofilm inducing factor. This suggests that herbal drug could be stronger inducer of biofilm than catecholamine *in vitro* and promote the ability of *K. pneumoniae* to cause infection. Further investigations into the untoward effect of biofilm production such as antibiotic resistance are necessary.

A number of people within rural and urban settings in Nigeria consume herbal solution some as a way of life while others for the purpose of eliminating infection. Consequentially, the observations from this investigation show the possibility of the effect of consumption of some herbal antimicrobial drugs by predisposing herbal drug consumers to opportunistic infection by enhancing *K. pneumoniae* biofilm formation. This encourages their colonization their survival in stressful situations. The clinical importance of this *in vitro* investigation is highlighted by the fact that it employed the same herbal solutions consumed by people in Nigeria together with the low inoculum of bacterial which represents the infectious dosage present during the initial stage of infection [16]. The findings in this study further buttress the observations in previous studies [17, 18] that herbal antimicrobial agents induce resistance, through suggesting that the production of biofilm could be a mechanism of resistance development employed by some herbal drugs.

4. CONCLUSION

This study was able to demonstrate for the first time that *in vitro* exposure of *K. pneumoniae* to herbal antimicrobial drugs could induce biofilm in *K. pneumoniae*. However, the mechanisms behind this biofilm induction are yet to be discovered.

CONSENT (WHERE EVER APPLICABLE)

This was not applicable in this research.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

This was not applicable in this research.

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