Journal of Advances in Microbiology

12(1): 1-7, 2018; Article no.JAMB.42685
ISSN: 2456-7116

Levels of Biofilm Expression in *Klebsiella pneumoniae* Isolates Exposed to Herbal Drugs

Monsi, Tombari Pius[1*], Abbey, Samuel Douglas[1], Wachukwu, Confidence Kinikanwo[1] and Wokem, Gloria Ngozika[1]

[1]Department of Medical Laboratory Science, Faculty of Science, Rivers State University, Nkpolu, Oroworukwo, P.M.B. 5080, Port Harcourt, Rivers State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. All authors designed the study, author MTP performed the statistical analysis, wrote the protocol. Authors MTP and ASD wrote the first draft of the manuscript. Authors WCK and WGN managed the analyses of the study. Author MTP managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2018/42685
Editor(s):
(1) Dr. Akpaka, E. Patrick, Professor, Unit of Pathology & Microbiology, Faculty of Medical Sciences, The University of the West Indies St. Augustine, Trinidad & Tobago.
(2) Dr. Pongsak Rattanachaikunsopon, Professor, Department of Biological Science, Faculty of Science, Ubon Ratchathani University, Thailand.
Reviewers:
(1) Masaaki Minami, Nagoya City University, Japan.
(2) Aynur Aybey, Bursa Uludağ University, Turkey.
(3) Yasmine Samy Moustafa Mohammed Ali, Tanta University, Egypt.
(4) Lucas Pizauro, Sao Paulo State University, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history/26184

Original Research Article

Received 12 June 2018
Accepted 24 August 2018
Published 11 September 2018

ABSTRACT

Background: There is a continuous rise in antimicrobial resistance globally. Factors responsible for this occurrence especially in developing countries are yet to be properly elucidated. Due to the financial implications of antimicrobials individuals in developing countries such as Nigeria resort to the consumption of herbal drugs to treat infections.
Aims: To investigate the levels of biofilm expressed in *Klebsiella pneumoniae* isolates pre-treated with herbal drugs.
Methodology: Biofilm assay was performed using 24-well polystyrene microtitre plates which mimic the surface for bacterial attachment. Control and clinical isolates of *K. pneumoniae* 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

**Corresponding author: E-mail: monsitp@live.com, tombari.monsi@ust.edu.ng;*

6.25%) in 24-well plate and incubated overnight at 37^{o}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 used to quantify the levels of biofilm attached to the surface of the 24-well plate. Results were anaylsed using 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 (50%) showed a 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* isolates

Conclusion: Bet, Gob and Ruz could predispose *K. pneumoniae* to enhance its production of biofilm.

Keywords: Biofilm; Klebsiella pneumonia; 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 the management of infections.

Antimicrobial resistance is a serious health concern as it impedes the management and prevention of infections. Different cases of antimicrobial resistance have been seen globally [2]. The emergence of resistant strains of tuberculosis has been observed in 4.5 million recent cases of antimicrobial resistant tuberculosis 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 of *K. pneumoniae* to carbapenem, a last resort antibiotic, is now noted in all parts of the globe [2].

Several 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 the same species or between different species. Multidrug resistance mechanisms occur naturally via erroneous replication or transfer of resistant traits [3]. The force driving this process is the selective force of antimicrobial utilisation. This is very notable in hospital environments where clear correlation between antimicrobial use and development of resistance can be seen [4,5,6].

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. Biofilms are surface-attached extracellular polysaccharide matrix. Bacteria form biofilm in order to successfully invade and damage the host tissue. 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 fold 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 of antimicrobial agent within the biofilm matrix [9] and presence of ''persister'' cells.

The aim of the current study was to examine the hypothesis that exposure of *K. pneumoniae* isolates to herbal treatments could increase the production of biofilm.

2. MATERIALS AND METHODS

2.1 Collection of Drugs

Locally-made herbal 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 of herbal drugs to dryness in test tubes. The differences in the weight of the test tubes after drying were determined. The weight differences obtained were: Goko Alcoholic bitters [Gab] (0.09 g/ml), Ruzu bitters [Ruz] (0.29 g/ml), Beta Cleanser [Bet] (0.09 g/ml), Goko Cleanser Herbal mixture [Gob] (0.09 g/ml).

2.3 Collection of Organisms

The laboratory strain (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 Culture media

The microbial media used were tryptone soya agar (TSA) and tryptone soya broth (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. [10]. *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 (without herbal drug supplementation) was performed alongside. The cultures (200 µL) were transferred into a 24-well polystyrene plate. 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 a photograph of the surface biofilm was 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 non-adherent 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 independent experiments. Data were analysed on Graph Pad Prism 5.0.

3. RESULTS

3.1 Cell-to-cell Attachment

K. pneumoniae isolates produced surface biofilm in Gob and Bet in the laboratory strain but only in Bet for the Control strain when viewed from the surface (Fig. 3.1). No surface biofilm were seen in Gab and Ruz. The two highest concentrations of all the drugs did not show any level of surface biofilm induction. The clinical strain showed a higher level of cell-to-cell aggregation in the Bet compared to the control.

3.2 Biofilm Analysis with Crystal Violet Assay

Fig. 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 measured in absorbance 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 (Figs. 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 a 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),

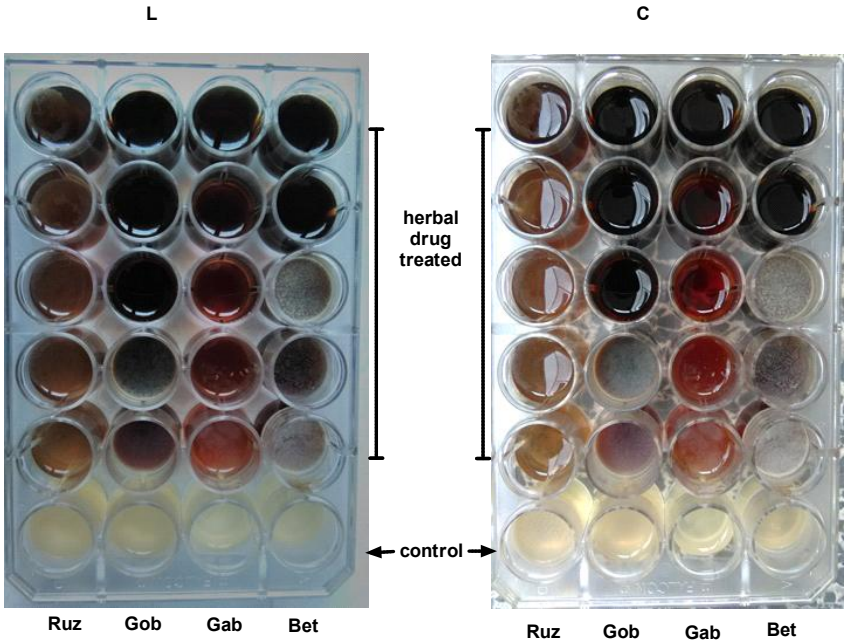

Fig. 3.1. Surface biofilm formation in *K. pneumoniae* isolates exposed to some herbal solutions

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

followed by Ruz (OD= 2.0), then Gab (OD= 1.5) and Gob (OD= 1.3). Figs. 3.2a and b showed a similar pattern of biofilm production: the 25% concentration showed much higher levels of optical densities. Bet (25%) and Ruz (50%) showed a significant level of biofilm formed compared to untreated control.

4. DISCUSSION

There are two ways biofilm can be formed in bacteria; cell-to-cell aggregation and attachment to surface [11]. The potential of bacteria to resist antibiotics and form a biofilm on medical devices is becoming high in hospital-acquired infections [10]. 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 causes of drug resistance in Nigeria.

The processes in bacterial biofilm formation initially begin by the first attaching to a surface [11]. Findings from other investigations have shown that pathogenic bacteria recognise inotropic drugs and use them to grow and produce biofilm [12,11]. However, information is yet available as to whether these herbal drugs induce biofilm in *Klebsiella* spp in a similar fashion. Hence, the aim of this study was to investigate biofilm levels in *K. pneumoniae* strains response to exposure to herbal drugs. In this investigation, it was shown that the 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 infection control and prevention. Biofilm formation in *K. pneumoniae* is an aspect of its pathogenicity that enhances the colonisation of a 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].

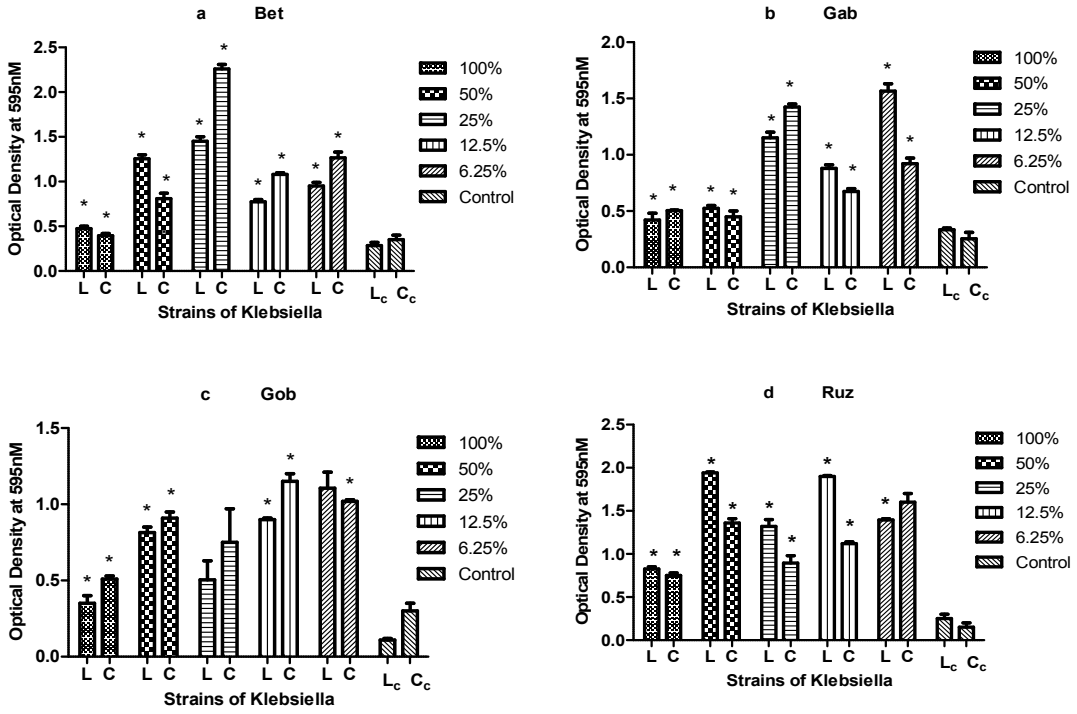

Fig. 3.2. Levels of expression of Biofilm in *K. pneumoniae*

*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*

Biofilm analysis of herbal drugs induction of biofilm observed in *K. pneumoniae* showed a minimum of two fold increase compared to control (Fig. 3.2a) and a maximum of 8-fold increase (Fig. 3.2d). A similar study by Freestone et al. [12] demonstrated that *Pseudomonas aeruginosa* another gram negative organism 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 also similar to the fold increase observed by Lyte et al. [10] using catecholamines as a biofilm inducing factor. This suggests that herbal drug could be a 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 solutions, some as a way of life while others for the purpose of eliminating infections. 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 infections by enhancing *K. pneumoniae* biofilm formation. The consumption habit of the herbal drugs by individuals promotes bacteria colonisation since the bacteria tend to survive more in stressful conditions. The clinical importance of this *in vitro* investigation is highlighted by the fact that it employed the same herbal drug 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 *K. pneumoniae* isolates produced biofilm when they were exposed to some herbal drugs. These findings further demonstrated the mechanism of antimicrobial resistance (biofilm production) observed in previous studies by Monsi et al. [17,18].

5. CONCLUSION

This study was able to demonstrate for the first time that *in vitro* exposure of *K. pneumoniae* to a herbal antimicrobial drug could induce biofilm in *K. pneumoniae*. However, the mechanisms behind this biofilm induction are yet to be determined and warrant further studies.

ACKNOWLEDGEMENT

The authors wish to acknowledge Tertiary education Trust Fund (TETFUND) for the financial support provided as grant to successfully complete this work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. Nature. 1940;146:837-837.
2. World Health Organization. Antimicrobial resistance: Global report on surveillance. World Health Organization; 2018.
   (Accessed 20 March 2018)
   Available:http://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance
3. Vadhana P, Singh BR, Bharadwaj M, Singh SV. Emergence of herbal antimicrobial resistance in clinical bacteria isolates. Pharmaceutica Analytica Acta. 2015;6(10):434.
4. Graffunder EM, Preston KE, Evans AM, Venezia RA. Risk factors associated with extended-spectrum β-lactamase-producing organisms at a tertiary care hospital. Journal of Antimicrobial Chemotherapy. 2005;56:139–145.
5. Lautenbach E, Weiner MG, Nachamkin I, Bilker WB, Sheridan A, Fishman NO. Imipenem resistance among *Pseudomonas aeruginosa* isolates: Risk factors for infection and impact of resistance on clinical and economic outcomes. Infection Control & Hospital Epidemiology. 2006;27:893–900.
6. Martinez JA, Aguilar J, Almela M, Marco F, Soriano A, Lopez F, et al. Prior use of carbapenems may be a significant risk factor for extended-spectrum b-lactamase-producing *Escherichia coli* or *Klebsiella* spp. in patients with bacteraemia. Journal of Antimicrobial Chemotherapy. 2006;58: 1082–1085.
7. Kaplan JB. Antibiotic-induced biofilm formation. International Journal of Artificial Organs. 2011;34(9):737-751.
8. Borriello G, Werner E, Roe F. Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. Antimicrobial Agents and Chemotherapy. 2004;48(7):2659-2664.
9. Singh R, Ray P, Das A, Sharma M. Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Journal of Antimicrobial Chemotherapy. 2010;65(9):1955-1958.
10. Lyte M, Freestone PP, Neal CP, Olson BA, Haigh RD, Bayston R, Williams PH. Stimulation of *Staphylococcus epidermidis* growth and biofilm formation by catecholamine inotropes. Lancet. 2003; 361(9352):130-135.
11. Conway BA, Chu KK, Bylund J, Altman E, Speert DP. Production of exopoly-saccharide by *Burkholderia cenocepacia* results in altered cell-surface interactions and altered bacterial clearance in mice. The Journal of Infectious Diseases. 2004; 190(5):957-966.
12. Freestone P, Hirst R, Sandrini S, Sharaff F, Fry H, Hyman S, et al. *Pseudomonas aeruginosa*-Catecholamine Inotrope Interactions: A contributory factor in the development of ventilator associated pneumonia? Chest. 2012;142(5):1200-1210.
13. Morehead RS, Pinto SJ. Ventilator-associated pneumonia. Arch Intern Med. 2000;160(13):1926-1936.
14. Garau J, Gomez L. *Pseudomonas aeruginosa* pneumonia. Curr Opin Infect Dis. 2003;16(2):135-143.
15. Ramirez P, Ferrer M, Torres A. Prevention measures for ventilator-associated pneumonia: A new focus on the endotracheal tube. Curr Opin Infect Dis. 2007;20(2):190-197.
16. Freestone PP, Lyte M, Neal CP, Maggs AF, Haigh RD, Williams PH. The mammalian neuroendocrine hormone norepinephrine supplies iron for bacterial growth in the presence of transferrin or

lactoferrin. Journal of Bacteriology. 2000; 182(21):6091-6098.

17. Monsi TP, Amala SE, Ugoaru NF. Acquisition of antibiotic resistance in *Escherichia coli* exposed to a locally produced herbal drug. Microbiology Research Journal International. 2017; 22(2):1-7.

18. Monsi TP, Wokem GN, Aleruchi PC. Development of antibiotic resistance in herbal drug-sensitized *Staphylococcus aureus* isolate. Journal of Advances in Microbiology. 2017;7(4):1–7.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/26184