CLINICAL RESEARCH

Occurrence and Antibiotics Resistance Signatures of *Burkholderia Pseudomallei* Isolated from Selected Hospital Final Effluents in Akoko Metropolis within Ondo State Nigeria

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ABSTRACT

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Introduction *Burkholderia pseudomallei* are the causative agent for melioidosis, a disease which affects both humans and animals. This study investigated the occurrence of *Burkholderia pseudomallei* in the final effluent of some hospitals in Akoko metropolis, Ondo State, Nigeria.

Methods Culture based approach using the Inositol Brilliant Green Bile agar for isolation *Burkholderia Pseudomallei* was employed. A preliminary oxidase strip test was carried out on all the isolates and they were found to be oxidase positive. Presumptive isolates were purified and confirmed using Microbact™ 24E Identification kit. Antibiotics susceptibility of the confirmed isolates was also determined using the CLSI recommended first line antibiotics for Burkholderia Pseudomallei.

Results A total number of 155 presumptive *Burkholderia* species were recovered from thirty six different samples over a period of three months from effluent of three selected hospitals. Moreover, 67% of the recovered isolates were confirmed to be *Burkholderia pseudomallei*. Most isolates were susceptible to cefotaxime and ofloxacin but exhibited resistance against tetracycline and meropenem.

Conclusions The persistence of *Burkholderia pseudomallei* in the hospital environment throughout the sampling regime requires intervention strategies to eradicate the prevalence of this notable pathogen in all possible reservoirs within the hospital environment. Besides, the emergence of resistance particularly to tetracycline and carbapenems points at potential public health implications. Furthermore, surveillance of *Burkholderia* species with its antibiogram profiles in clinical environments and adequate treatment of hospital effluents before disposal is very important to avert potential outbreak of melioidosis because the main reservoir for *B. pseudomallei* is contaminated environments.

Keywords Melioidosis - effluents - antibiotics - *Burkholderia pseudomallei.*

INTRODUCTION

The order *Burkholderiales* has three well-known families. *Burkholderia* is placed in the family *Burkholderiaceae*. Members of this genus are Gram-negative, aerobic, non-fermentative, non-spore forming, mesophilic straight rods.[1] With the exception of *Burkholderia mallei,* all members are motile with a single polar flagellum or a tuft of polar flagella. Most species use poly-β-hydroxybutyrate as their carbon reserve.[2] The organism is associated cystic fibrosis patients.[3] *Burkholderia pseudomallei* is a soil saprophyte and the causative agent of melioidosis, an infectious disease that is predominantly restricted to Southeast Asia and northern Australia, and is increasingly recognized in other tropical and subtropical regions.[4] *Burkholderia pseudomallei* is an environmental bacterium, infecting humans by contact with contaminated soil or water.[5] In areas where the disease is endemic, the organism is commonly found in soil and surface water.[6] Infections occur mostly by inoculation, ingestion and inhalation of the organism through puncture wounds in the skin and seldom through person-to-person, sexual, perinatal, vertical, and nosocomial routes.[7] *B. pseudomallei* are an opportunistic pathogen capable of infecting humans[8] and a large variety of animals.[9] Furthermore, immuno-compromised individuals with diseases such as diabetes, viral infection, cystic fibrosis, hazardous alcohol use, chronic renal disease and those undergoing immunosuppressive therapy are at particular risk of acquiring and dying from melioidosis.[10] Clinical presentations vary widely and it includes skin and soft tissue abscesses, pneumonia and disseminated infection with septic shock.

Pollution of aquatic ecosystem especially freshwater resources with anthropogenic contaminants is a major concern of interest because the available freshwater resources is needed for drinking, domestic use, food production, and recreational purposes. Freshwater resources are contaminated through the indiscriminate discharge of untreated hospital effluent and other industrial waste into the natural environment.[11] The situation is particularly alarming in under-develop and developing regions such as in Sub-Saharan Africa where most rivers, lakes, and lagoons are receiving untreated hospital effluent.[12,13] Hospital effluents consist of complex mixtures of chemical and biological substances which are continually discharged into the environment which in turn sink to the underground water and contaminate aquatic ecosystem.[14] In addition, hospital effluents is the outcome of diagnostic laboratory and research activity waste and medicine discharge which include active components from medicinal products and their metabolites, chemicals, disinfecting agents, specific detergents, radioactive substances, iodinated contrast media, nutrients, bacteria and their antimicrobial resistance genes.[15] Disposal of untreated hospital effluent, infectious waste and equipment can decrease the quality of the receiving waterbodies.[16]

Uncontrolled and excessive use of antibiotics by human and animals results in an increase in antibiotic resistance which leads to spread of resistance genes in environmental samples such as hospital effluent.[17] *B. pseudomallei* naturally resist a large array of antimicrobial agents, such as macrolides, narrow-spectrum cephalosporins, most penicillins, polymyxins, and aminoglycosides. Moreover, clinical evidence indicates that fluoroquinolones are also ineffective.[18] Hospital effluent is still daily discharged into surface water in the Akoko Metropolis within Ondo State Nigeria and therefore the essence of this monitoring study on the occurrence and antibiotics resistance signatures of Burkholderia Pseudomallei in the hospital effluent. Hence, this study is designed to investigate the occurrence of *Burkholderia pseudomallei* in the final effluents of some hospitals in Akoko metropolis of Ondo state, Nigeria.

METHODOLOGY

Study Area

The study focused on health facilities that discharge their final effluents into their immediate environment. Thus, the research was carried out at sites (2 hospitals and 1 health Centre) to collect the final effluent namely; Hospital A: Akungba Akoko (Outlet A and B); Hospital B: Iwaro, Oka-Akoko (Outlet A and B) and Hospital C: Ikare Akoko (Outlet A and B) all in Ondo state, Nigeria. The consent to use the final effluent from the three hospitals for this study was sought and approved by the management of respective hospitals.

Sample Collection, total culturally bacterial density and isolation of presumptive *Burkholderia pseudomallei*

The hospital effluent was collected from each of the sampling points into sterile glass sampling bottles and transported to the laboratory for analysis. At the laboratory, total culturable bacterial count was determined and the samples were analyzed for the presence of *Burkholderia pseudomallei* using Inositol Brilliant Green Bile Agar as the selective medium and peptone water as enrichment medium. A ten-fold serial dilution was carried out on each of the samples up to dilution factor four using normal saline solution as diluent. To determine total bacterial count per sample, 100 µL aliquot from each dilution tubes was plated on sterile nutrient agar plate, incubated at 37 ^{o}C and distinct colonies were counted after 24 hours of incubation. Also, 1 mL aliquots from each dilution tubes were introduced into test-tube containing 9 mL of sterile APW which serves as enrichment medium for *B. pseudomallei*. The tubes were incubated at 37 ^{o}C for 24 hours and

a loop full from each tube was streaked on fresh sterile Inositol Brilliant Green Bile agar plates after the incubation period. The streaked plates were also incubated for 24 hours at 37ºC and representatives of all distinct and unique colonies were selected as presumptive isolates. Each of the selected colonies were further streaked on sterile nutrient agar plates and the consistency of the resulting colonies were checked to ascertain the purity of each of the colonies picked from Inositol Brilliant Green Bile agar. The purified presumptive isolates were stored in 25% glycerol at 4ºC for further analysis.

Identification of the Bacteria Isolates

All presumptive isolates were initially subjected to oxidase test using oxidase strips to screen out oxidase negative isolates since all *B. pseudomallei* are oxidase positive. All oxidase positive isolates were identified using Microbact™ 24E Identification Kit afterwards by following manufacturer instructions. Standardized 24 hours old broth culture of each of the isolates was prepared and 50µl of the culture suspension was dispensed into every well on the kit. Wells meant for anaerobic incubation were overlaid with mineral oil and the kit was incubated for twenty-four hours at 37º C following manufacturer specification. After the incubation period, appropriate reagents (Indole, VPI, VPII, TDA, NIT A/B and mineral oil) were added to wells and colour change was observed, interpretation was done according to a colour chart standard provided by the kit manufacturer and were recorded on result forms to generate result codes. The generated result codes were later entered into the Microbact software which returns the most likely organism based on similarity index.

Antimicrobial Susceptibility Testing

The disk diffusion susceptibility method was used to test the resistance of *Burkholderia pseudomallei* against selected antibiotics under aseptic condition. This test was carried out by preparing a bacterial suspension whose turbidity is equivalent to McFarland standard of 0.5 ml, and swabbing the bacterial suspension of approximately 1.5 x10^8 cfu/ml evenly on the surface of already prepared sterile Mueller Hinton agar plate. Six commercially prepared with fixed concentration, paper antibiotic disks were dispensed on the agar surface using antibiotic dispenser. The plates were incubated for 18-24 hours at 35-37 º C before the results were determined. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter. The zone diameters of each antibiotic were interpreted as described by the Clinical and Laboratory Standards Institute.[19]

RESULTS

A total of 155 presumptive *Burkholderia pseudomallei* isolates were recovered from 2 hospitals and 1 health centre effluent collected from six hospital sampling sites. On the basis of morphological and cultural observation, some of the presumptive isolates were pinkish in colour and others appeared white in colour on Inositol Brilliant Bile Green agar. The distribution of the presumptive isolates among the effluents from the sampling points was 74 (48%), 60 (39%) and 21 (13%) for Akungba health Centre, Iwaro-Oka hospital, and Ikare Akoko hospital respectively. All the presumptive isolates were oxidase positive but only 68% of the isolates were confirmed as *Burkholderia pseudomallei* with the aid of Microbact TM 24E (Oxoid UK). All the confirmed isolates were pinkish in colour on Inositol Brilliant Green Bile agar. Some of the result test code, Probable Identity and Percentage accuracy of identity of the representative isolates using Microbact™ 24 E (Oxoid UK) Identification Kit is shown in table 1, while Figure 2 also shows a representative sample of Microbact™ 24E (Oxoid UK) identifying five out of the confirmed *Burkholderia pseudomallei* isolates. Distribution analysis of antimicrobial susceptibility profile of Burkholderia Pseudomallei is presented in Figure 3 and 4. *Burkholderia Pseudomallei*, 30% were resistant to Gentamicin, 90% were resistant to Tetracycline, 100% were resistant to Meropenem and 20% were resistant to trimethoprim sulfamethoxazole.

Table 1 Identification of five isolates using Microbact™ 24 E (Oxoid UK) Identification Kit

Isolates	Result Test Code	Probable Identity	% probability
ERA1	645573751	*Burkholderia pseudomallei*	99.95%
ERA 2	647773765	*Burkholderia pseudomallei*	98.12%
ERA 3	647753776	*Burkholderia pseudomallei*	91.24%
ERA 4	647773755	*Burkholderia pseudomallei*	99.89%
ERA 5	645773776	*Burkholderia pseudomallei*	90.04%

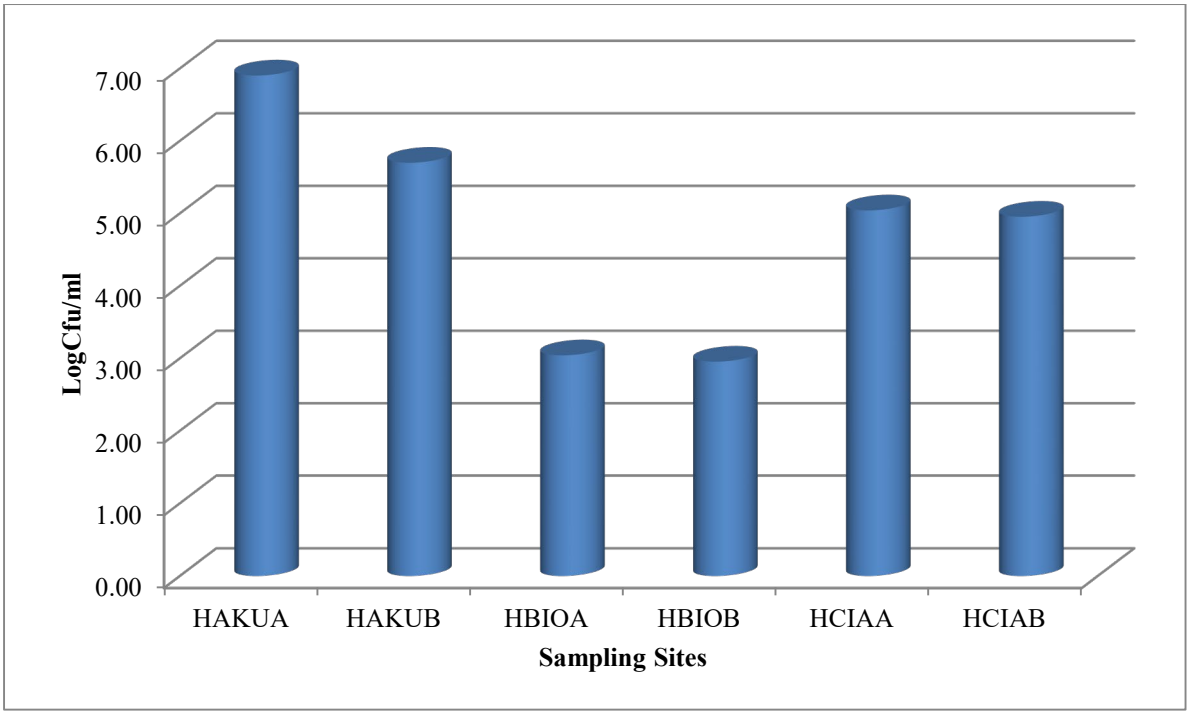


Figure 1 Distribution of *Burkholderia pseudomallei* in 2 hospitals and 1 health centre (HAKUA: Hospital A, Akungba (A); HAKUB: Hospital A, Akungba (B); HBIOA: Hospital B, Iwaro- Oka, Akoko(A); HBIOB: Hospital B, Iwaro- Oka, Akoko (B); HCIAA: Hospital C, Ikare- Akoko (A); HCIAB: Hospital C, Ikare- Akoko (B))

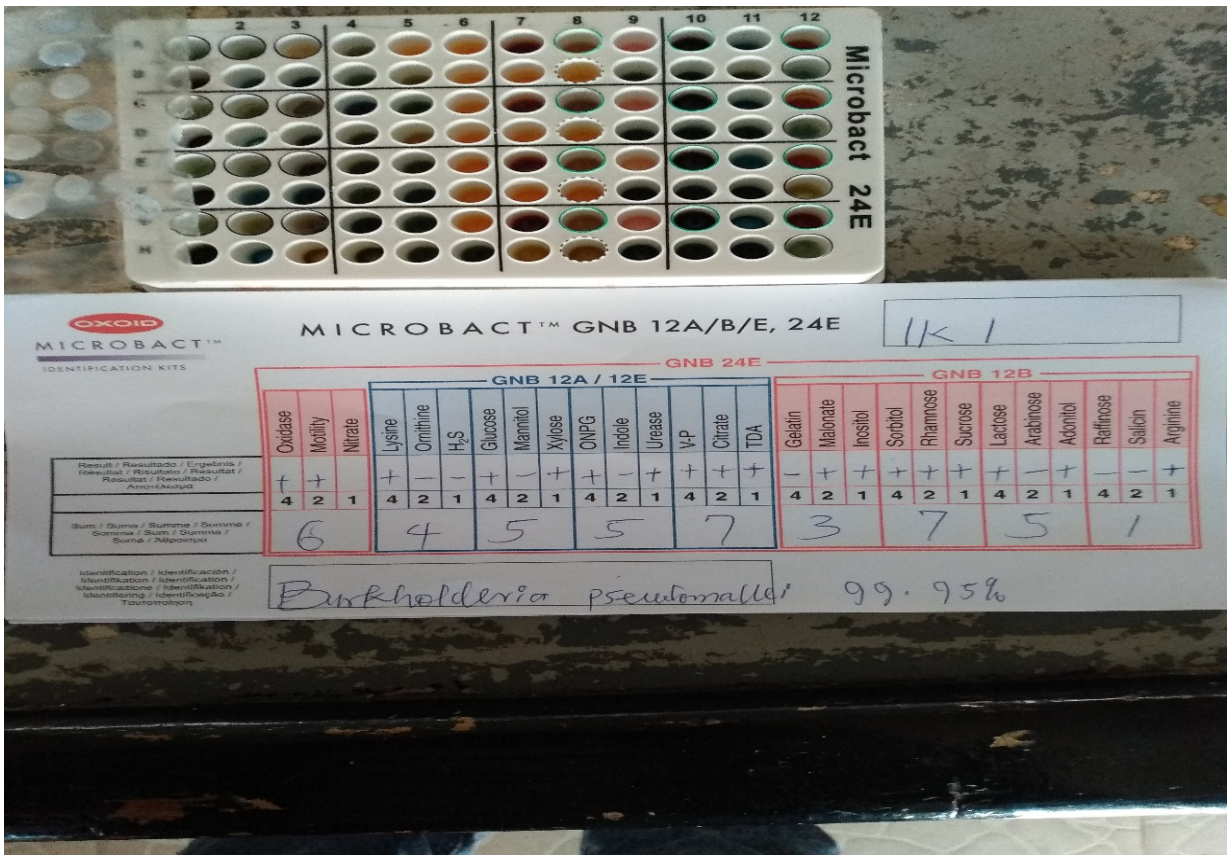
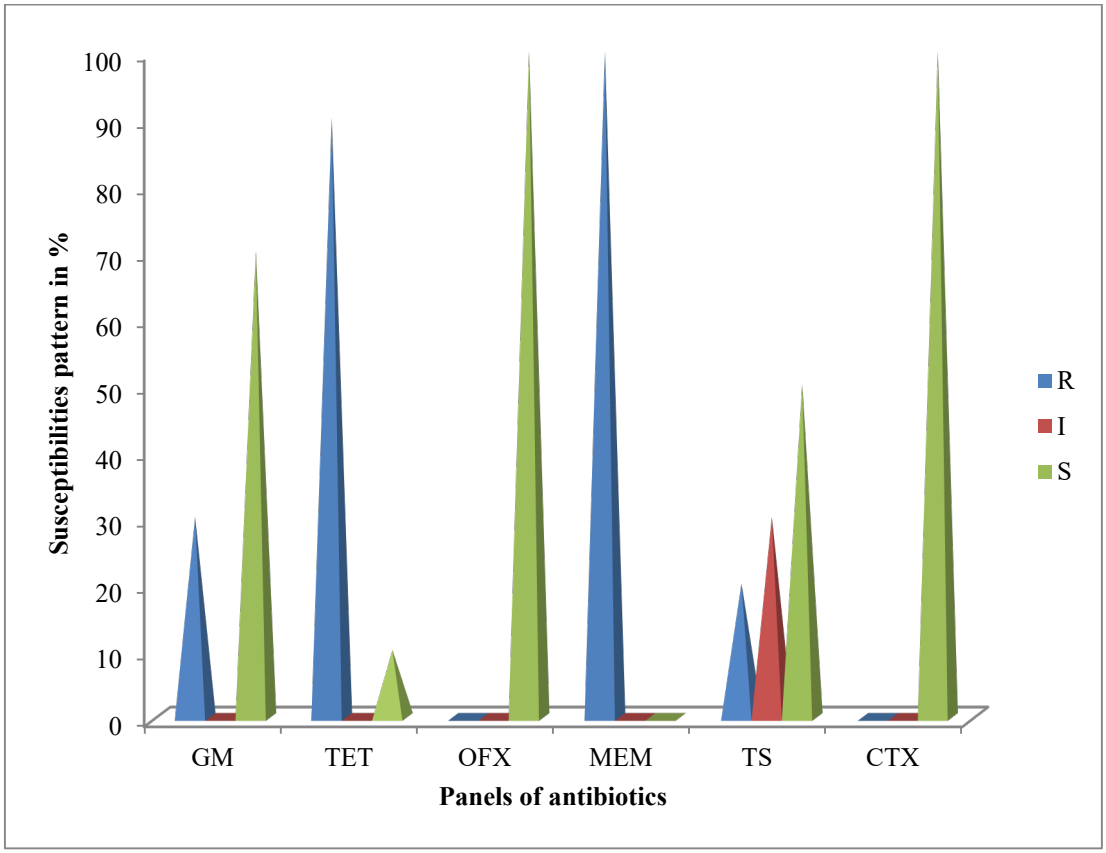


Figure 2 Identity of the microorganisms using MicrobactTM 24E (Oxoid UK)

Table 2 Information and Interpretive Criteria for Susceptibility testing

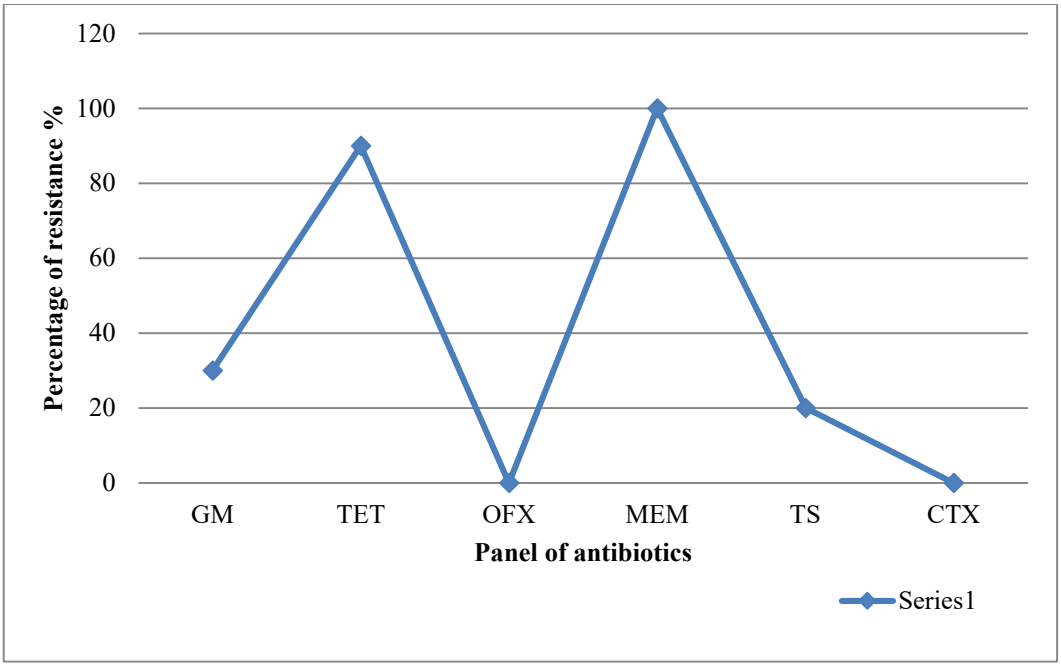
S/N	Antimicrobial Class	Antimicrobial agent	Conc. µg	MIC (µg/mL) Interpretive criteria S	I	R
1	Aminoglycosides	Gentamicin	10	≥15	13-14	≤12
2	Tetracycline	Tetracycline	30	≥19	15-18	≤14
3	Quinolones	Ofloxacin	5	≥16	13-15	≤12
4	Carbapenem	Meropenem	10	≥20	16-19	≤15
5	Folate Inhibitors	Trimethoprim sulfamethazole	1.25	≥16	11-15	≤10
6	Cephems	Cefotaxime	30	≥21	18-20	≤17

Notes: Breakpoints as recommended by the CLSI (2017). S, I and R stand for susceptible, intermediate and resistant, respectively.



Notes: R- Resistance, I- Intermediate, S- Susceptiblity, GM- Gentamicin, TET- Tetracycline, OFX- Ofloxacin, MEM- Meropenem, TS- Trimethoprim sulfamethazole, CTX- Cefotaxime

Figure 3 Antibiotic susceptibility profiles of confirmed *Burkholderia pseudomallei* isolated from Hospital effluents in Akoko Metropolis, Ondo State.



Notes: GM- Gentamicin, TET- Tetracycline, OFX- Ofloxacin, MEM- Meropenem, TS- Trimethroprim sulfamethazole, CTX- Cefotaxime

Figure 4 Resistance profiles of confirmed Burkholderia pseudomallei recovered from Hospital Effluent in Akoko Metropolis, Ondo State.

DISCUSSION

The result of this study showed that effluents of each of 2 hospitals and 1 health centre have common source of microbial contamination. The Inositol Brilliant Green Agar, a selective medium for selective isolation of *Pleisomonas shigelloides* (an opportunistic pathogen) and *Aeromonas* species was used in this study as an alternative to Ashdown medium, the recovered isolates presents the same distinct colonies with typical morphological characteristics (pink colonies) similar to *Pleisomonas shigelloides.* After obtaining the pure isolates, 155 isolates were all confirmed to be oxidase positive; this result was in accordance with a study on *Burkholderia pseudomallei* isolates from coastal region in India.[20] Further confirmation with Microbact™ 24E (Oxoid UK) identification Kit signifies *Burkholderia pseudomallei* for all the recovered isolates. Our findings showed that Inositol Brilliant Green Agar is preferable for isolating *Burkholderia pseudomallei*, especially when result is required within 24-48 hours because both contain bile salt mixture which inhibits the growth of Gram positive bacteria, peptone, meat extract and yeast extract which supply nitrogenous nutrients required for the growth of the desired microorganisms.[21] Identification of *Burkholderia pseudomalleii* from the hospital effluent suggests that patient with the disease are shedding it into the hospital milieu and that the treatment plan of the hospitals for effluents is not sufficient to eliminate the pathogen from the effluent before they are discharge into the environment. *B. pseudomallei* also poses a worldwide emerging infectious disease problem and a bioterrorism threat due to its severe course of infection, aerosol infectivity, low infectious dose, an intrinsic resistance to commonly used antibiotics, lack of a currently available vaccine, and the worldwide availability.[22]

This is of great concern to the public health since the pathogen has myriad of intrinsic antibiotics resistance ability making its infection difficult to treat.[23] All the confirmed *Burkholderia pseudomallei* isolates were susceptible to cefotaxime and ofloxacin, while 70% and 50% of the isolates were susceptible to gentamicin and trimethoprim sulfamethazole respectively. Species within this group have been established as organisms of concern in both environmental and nosocomial settings, with members of the *Burkholderia* genera already known to be intransigent to standard first-line therapy as a result of both acquired and intrinsic resistance factors.[24,25]

On the other hand, 90% and 100% of the isolates demonstrated resistance against meropenem and tetracycline respectively. Antibiotics susceptibility results of this study was not in agreement with the findings of [26] who described the use of Ceftazidime and Carbapenem which are members of the third generation cephalosporin antibiotics as the effective treatment for meliodosis.[26] As much as it seems that the result of the susceptibility of the confirmed *Burkholderia pseudomallei* to meropenem seems odd, it is not out of place because recent findings has shown the

possibility of isolating meropenem-resistant *Burkholderia pseudomallei* from clinical environments.[26,27] *Burkholderia pseudomallei* found to be resistant to tetracycline and meropenem from this study could be attributed to its ability to produce β-lactamases enzyme that is capable of hydrolyzing the β-lactam ring of the antibiotics (tetracycline and meropenem).

The study has also shown that *Burkholderia pseudomallei can* be isolated from environmental samples such as untreated hospital's effluent. The study calls for a proper treatment of hospital effluents to ensure that they are devoid of pathogen such as *Burkholderia pseudomalleii* before they are discharged into the environment. To the best of our knowledge no report has documented the occurrence and antibiotics resistance signatures of *Burkholderia pseudomalleii* in hospital effluent in Akoko Metropolis, where it had never been previously detected in the environment or in humans. The isolation of this important bacterial species from this part of Nigeria should initiate further studies on the extent of environmental and clinical impact of melioidosis in Nigeria. Finally, the two most effective antibiotics (Cefotaxime and Ofloxacin) against *Burkholderia pseudomallei* as observed in this study could be considered as drug of choice in case melioidosis outbreak management.

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