Phenotypic Characterization of *Burkholderia Pseudomallei* Isolates In Fishes In FCT, Abuja Using Microbact™ GNB 24E Identification Test Kit System.

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Abstract: **The study was carried out to determine the phenotypic characters of *Burkholderia pseudomallei* isolates from fresh water fishes sold in FCT using culture, isolation and complete biochemical identification followed by confirmation with Microbact™ GNB24E identification system. Out of a total number of about 400 samples collected from different fishes (257 from *Tilapia zillii,* 77 from *Clarias gariepinus,* 58 from *Lates niloticus,* and 8 from *Alestes nurse*). Biochemical characterization revealed that 40 isolates were confirmed as *Burkholderia pseudomallei*. All the isolates (100 %) were positive to, catalase, hydrogen sulphide, voges proskauer and motility tests others showed varying percentage of reactions to other biochemical tests. The isolates were then subjected to confirmatory test using the Microbact™ GNB 24E kit which revealed that 25 isolates were *Burkholderia pseudomallei.* The results were subjected to descriptive statistics using percentages, ratios and tables. *Burkholderia pseudomallei* isolated had an overall prevalence rate of 6.25 %. The prevalence rate among fishes were *Alestes nurse* (50 %), *Lates niloticus* (11.11%) and *Tilapia zillii* (7.60%). Based on spread in various areas in Federal Capital Territory (FCT) , *Burkholderia pseudomallei* was higher in Bwari (8.75 %), followed by Gwagwalada with 8.33% and lower in AMAC (5.34%). This research is the first to expose *Burkholderia pseudomallei* from fishes in Gwagwalada. There is need for radical studies on this organism in other to determine its role as a biological chemical weapon in Nigeria.**

Keywords: ***Burkholderia pseudomallei*** **, fishes, phenotypic, characterization.**

I. INTRODUCTION

Burkholderia pseudomallei formerly known as *Pseudomonas pseudomallei*, is a gram-negative, bipolar, aerobic, motile rod-shaped bacterium belonging to the family Burkholderiaceae, it is a soil-dwelling bacterium endemic in tropical and subtropical regions worldwide [9]. *Burkholderia pseudomallei* was discovered in 1911 by Whitmore and his team [18]. Many years later, this bacterium was proven to cause melioidosis [8]. Melioidosis is an important cause of illness and death in humans and animals [5]. Melioidosis usually occur as a rapidly progressing septicemia with or without pneumonia, a localized soft-tissue infection, or a subclinical infection with delayed conversion to a clinically evident infection [7]. The routes of transmission of *Burkholderia pseudomallei* include inoculation via skin abrasion, inhalation, and ingestion [2].

Many terrestrial and aquatic mammals, as well as marsupials, birds, reptiles and fish, can be affected by melioidosis [11]. Goats, sheep and pigs are the most commonly infected species in Australia; sheep and goats seem to be particularly susceptible to clinical signs. Cases of melioidosis have also been reported in other species including dogs, cats, cattle, buffalo, camels, alpacas, horses, mules, bison (*Bison bison*), zebra (*Equus burchelli*), deer, kangaroos, wallabies, koalas, hog badger (*Arctonyx collaris*), large felids (e.g., cheetah, *Acinonyx jubatus*, and flat-headed cat, *Prionailurus planiceps*), various non-human primates, captive marine mammals, crocodiles, snakes, iguanas and tropical fish [17]. This disease has been documented in some species of birds including psittacine birds, penguins, ratites and chickens. Some reports suggest that, among birds, species not native to endemic regions may be more likely to develop melioidosis [15]. Rodents and rabbits can be infected experimentally.

Humans are susceptible to *B. pseudomallei*. This organism is usually acquired from environmental sources, but a few zoonotic cases have been described [11]. *Bukholderia pseudomallei* measures 2–5 μm in length and 0.4–0.8 μm in diameter and is capable of self-propulsion using flagella. The bacteria can grow in a number of artificial nutrient environments, especially betaine- and arginine-containing ones. Indigenous cases in animals and humans or organisms in the environment have been documented in many regions of the world such as Australia, Africa, Asia, America [1].

Burkholderia pseudomallei have been identified as a potential biological weapon [16].

[14] noted that *Burkholderia pseudomallei* can be reliably identified using commercial kit system for bacterial identification. The use of commercial identification kit is now becoming useful since it provides more accuracy and exposes definitive strains that are present in a given area. This research provides the first hand information on the use Microbact 24E identification kit to confirm *Burkholderia pseudomallei*. One of the best methods for diagnosis of the disease is Strauss test method, but due to certain inadequacies, or limitations associated with the test. There is need to isolate the organism. To the best of our knowledge this is the first research in FCT that cultured and isolated *Burkholderia pseudomallei* using the Microbact™ 24E GNB identification system. Our research

is useful as it provides knowledge for the molecular characterization of this organism. This research work was therefore aimed at determining the phenotypic characters of *Burkholderia pseudomallei* isolates from fresh water fishes in Federal Capital Territory, Abuja Nigeria.

II. MATERIALS AND METHODS

Sample processing

The collected fish sample was kept in dorso ventral position and the abdomen was then washed with 70% ethanol to reduce the number of incidental organism. A deep ventral incision was made and transverse incision was conducted to expose the visceral organ using a sterilized scissors, scapel and forcep, 5g of fish intestine was weighed and introduced into 45mls of peptone water as pre enrichment media, and then stomached using a stomacher for about 2minutes to homogenize the sample, after which 1ml was taken and transferred into labeled test tubes containing 9mls of already prepared alkaline peptone water or brain heart infusion broth and covered quickly and then incubate at 37^{O}C for 24 hours [7].

Media preparation

Agars will be prepared based on the manufacturer's instruction (Oxoid England, and Hi media India).

Ampicillin sheep blood agar: 40g of blood agar base was suspended in 900mls of distilled water, allowed to dissolve completely by boiling and then sterilize by autoclaving at 121^{o}C for 15 minutes. Allowed to cool to about 45 – 60 ^{o}C. 5% sheep blood agar (50mls) was then added and 4mls of ampicillin supplement was added.

Enrichment

Brain heart infusion broth was used for enrichment. 37grams was suspended in 1 litre of distilled water, boiled to completely dissolve and 5mls were dispensed into each enrichment bottle autoclaved at 121 ^{o} C for 15 minutes and allowed to cool. 1ml from the pre enrichment was transferred to 9 mls of brain heart infusion broth and then incubated at 37°C for 18 hours.

Selective plating

A loopful of the inoculum from the incubated test tubes of brain heart infusion broth was taken and streaked on Ampicillin sheep blood agar plates, the plates were incubated at 37°C for about 18-24 hours and smooth, convex, grayish colonies was isolated.

Preliminary identification

Preliminary identification was done by carrying out gram staining, oxidase test and catalase test. Gram negative, catalase positive isolates was stored on blood agar slant for further biochemical characterization [3].

Conventional Biochemical characterization

Biochemical test such as hydrogen sulphide, motility test, indole test, methyl red test, citrate test, DNase test, urease test, Voges proskauer test, sugars (glucose, inositol and mannitol, was performed [3].

Confirmation of Burkholderia pseudomallei using Microbact^{TM} GNB 24 E identification kit

Microbact^{TM} GNB 24 E identification kit is used in the identification of aerobic and facultative anaerobic gram-negative bacteria [6]. It contains 24 biochemical substrates, lysine, ornithine, hydrogen sulphide, glucose, mannitol, xylose, ONPG, indole, urease, Voges proskauer, citrate, tryptophan, gelatin, malonate, inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose, salicin, arginine.

Preparation of Inoculum

A single colony of 18- 24 hour's culture was picked using an inoculating loop and then emulsified in 5mls of already prepared normal saline (0.85g into 100mls of distilled water allowed to dissolve and then autoclaved), and then mixed thoroughly to get a homogenous suspension.

Inoculation

The plates were numbered first and the well of individual substrate was exposed by peeling its back. The plates were then placed on the holding tray. A sterile micro pipette was used to add 100ul of bacterial suspension to each well.one drop of mineral oil was added to well 1, 2, and 3. The inoculated rows was resealed with the adhesive seal and the incubated at 37^{o}C for 18-24 hours. After incubation the strips was removed from the incubator and the sealing tape was pealed backward. For well 8 (indole production) 2 drops of kovac's reagent was added and the result was read after 2 minutes of the addition of the reagent.

For well 10 (voges proskauer reaction) one drop of VP1 and VP2 reagent was added and the result was read after 15- 30 minutes. For well 12 (Tryptophan Deaminase) one drop of the TDA reagent will be added and the result was read immediately. For well 7 (ONPG), after reading the result, one drop of nitrate A and nitrate B reagent was added to the well. Production of red colour indicates that nitrate have been reduced to nitrite (NO_2). For wells that exhibited a yellow colour after the addition of nitrate reagents, Zinc powder was added to the well to determine if nitrate was reduced completely to nitrogen gas (N_2)

Interpretation: The codes gotten from the reaction was entered into Microbact computer aided identification package software and the resulting identity of the organism and its percentage probability was recorded.

III. RESULTS

Table one (1) shows the number of fishes purchased from different area councils in FCT Abuja. The highest number of *Tilapia Zillii*, which is 129 was purchased from AMAC, while

92 *Tilapia zillii* was purchased from Bwari area council and 36 *Tilapia zillii* from Gwagwalada area council, making a total of 257 *Tilapia zillii*. The highest *Clarias gariepinus* was purchased from AMAC which was about 70 and 7 was purchased from Gwagwalada area council respectively, no *Clarias gariepinus* was purchased from Bwari area council. 40 *Lates niloticus* were purchased from AMAC and 9 each was purchased from Bwari and Gwagwalada area council, making a total of 58 *Lates niloticus.* 6 *Alestes nurse* were purchased from AMAC and 2 from Bwari area council making a total of 8 *Alestes nurse.*

Table two (2) shows he dissipation of various complete biochemical characterization of 40 isolates suspected to be *Burkholderia pseudomallei* specie out of 400 fish samples used based on morphology, gram staining and complete biochemical test. All the 40 isolates were gram negative, greyish, moist, circular, and raised on Ampicillin sheep blood aagr. All the 40 isolates were positive for oxidase, catalase, hydrogen sulphide production, voges proskauer, and were all motile. 39 isolates fermented glucose, while 38 isolates fermented mannitol. Other reactions were as follows; urease production was positive for 37 isolates, methyl red was positive for 34 isolates, 24 isolates were also positive for indole and 18 isolates were positive for inositol.

Table three (3) shows the result of the 25 isolates confirmed by Microbact^{TM} GNB 24E to be *Burkholderia pseudomallei.* After subjecting the 40 suspected isolates of *Burkholderia*, Microbact^{TM} GNB 24E confirmed 25 isolates to be *Burkholderia pseudomallei.* The isolates gave various reactions as follows: All the 25 isolates were positive for motility, nitrate, hydrogen sulphide, xylose, glucose, rhamnose, sucrose, and arginine. 24 of the isolates reacted to lysine, mannitol, urease, citrate, mannose, 23 were positive to sorbitol, 22 were positive to raffinol, arabinose, voges proskauer, 21 isolates were positive to ONPG, inositol, and lactose, 20 isolates were positive to ornithine,16 were positive to salicine, 10 positive to adonitol and 3 positive to TDA. No isolate was positive to indole.

Table 4 shows the prevalence of *Burkholderia pseudomallei.* in different area councils of FCT, Abuja. The prevalence of *Burkholderia pseudomallei.* from fishes purchased in Bwari was the highest at (8.73 %) when compared to the prevalence of *Burkholderia pseudomallei* from fishes purchased in AMAC (5.34%) and Gwagwalada (8.33 %). The overall prevalence of *Burkholderia pseudomallei* isolates from the 400 fishes purchased in FCT was therefore 6.25 %.

Table 5 shows the percentage distribution of *Burkholderia pseudomallei.* among fishes purchased in Bwari. The highest isolation rate is for *Alestes nurse* (50 %) followed by *Lates niloticus* (11.11 %) and *Tilapia zillii* having the lowest (7.60 %), no isolation was recorded for *Clarias gariepinus* bringing the total isolation rate of *Burkholderia pseudomallei* in Bwari to 8.73%.

Table 6 shows the percentage distribution of *Burkholderia pseudomallei* among fishes purchased from AMAC. *Alestes nurse* had the highest isolation rate (16.66 %) followed by *Clarias gariepinus* (8.57 %) and then *Late niloticus* (10.0 %). *Tilapia zillii* had the lowest isolation rate of 1.55 %.

Table 7 shows the percentage distribution of *Burkholderia pseudomallei* among fishes purchased from Gwagwalada. Gwagwalada had an overall prevalence rate of 5.76 % with *Tilapia zillii* having 8.33% and no prevalence rate was recorded for *Clarias gariepinus, Alestes nurse,* and *Lates niloticus.*

Table 1: Fishes Purchased from different Area Councils in FCT, Nigeria

Types of Fish	AMAC	Bwari	Gwagwalada	Total
Tilapia zillii	129	92	36	257
Clarias gariepinus	70	-	7	77
Lates niloticus	40	9	9	58
Alestes nurse	6	2	-	8
Total	243	103	52	400

Table 2: Biochemical reactions of the 40 suspected isolates of *Burkholderia pseudomallei*

Biochemical test	No. of Positive	No. of Negative
Oxidase	40	0
Catalase	40	0
H_2S	40	0
VP	40	0
Motility	40	0
Glucose	39	1
Mannitol	38	2
Urease	37	3
Citrate	36	4
Methyl red	34	6
Indole	24	16
Inositol	18	12

Table 3: Confirmation Result of the 25 *Burkholderia pseudomallei* isolates using Microbact^{TM} GNB 24E identification kit.

Biochemical	Positive	Negative
Motility	25	0
Nitrate	25	0
Lysine	24	1
Ornithine	20	5
H_2S	25	0
Glucose	25	0
Mannitol	24	1

Xylose	25	0
ONPG	21	4
Indole	0	25
Urease	24	1
VP	22	3
Citrate	24	1
TDA	3	22
Gelatin	10	15
Mannose	24	1
Inositol	21	4
Sorbitol	23	2
Rhamnose	25	0
Sucrose	25	0
Lactose	21	4
Arabinose	22	3
Adonitol	10	15
Raffinol	22	3
Sal	16	9
Arginine	25	0

Table 4: Prevalence of *Burkholderia pseudomallei* in different area councils of FCT, Abuja.

Area councils	Numbers of fishes used	Number of isolates	%Prevalence
Bwari	103	9	8.73
AMAC	243	13	5.34
Gwagwalada	52	3	8.33
Total	400	25	6.25

Table 5: Percentage distribution of *Burkholderia pseudomallei* among fishes in Bwari.

Types of fishes	Number of fishes	Number of isolate	Prevalence rate%
Tilapia zillii	92	7	7.60
Lates niloticus	9	1	11.11
Alestes nurse	2	1	50.00
Clarias gariepinus	0	0	0.00
Total	103	9	8.73

Table 6: Percentage distribution of *Burkholderia pseudomallei* Among Fishes in AMAC

Types of fish	Number of fishes	Number of isolates	Prevalence rate%
Tilapia zillii	129	2	1.55
Clarias gariepinus	70	6	8.57
Lates niloticus	40	4	10.00
Alestes nurse	6	1	16.66
TOTAL	243	13	5.34

Table 7: Percentage distribution of *Burkholderia pseudomallei* Among Fishes in Gwagwalada

Types of fish	Number of fishes	Number of isolates	Prevalence rate%
Tilapia zillii	36	3	8.33
Clarias gariepinus	7	0	0.00
Lates niloticus	9	0	0.00
Alestes nurse	0	0	0.00
TOTAL	52	3	5.76

IV. DISCUSSION

Isolation of Burkholderia specie

Burkholderia specie is capable of growing on the common culture media used in the clinical laboratory, such as Sheep Blood Agar (SBA), as well as Chocolate Agar and Mac conkey agar. Although selective media for the isolation of *Burkholderia pseudomallei* is the ashdown's media which is often used for the effective isolation of the organism. Mature colonies of *B. pseudomallei* on solid agar media often take on a wrinkled appearance after several days of incubation in solid media [6]. Some strains do not show this wrinkling effect, which is more pronounced in solid agar formulations containing glycerol as noticed in this study. *Burkholderia pseudomallei* are motile, particularly in the early stages of their growth cycle. Some strains of *Burkholderia pseudomallei* produce smooth colony growth on first culture and occasional strains are overtly mucoid, with an appearance similar to that of *Pseudomonas aeruginosa* capsular polysaccharide overproducers [13]. As observed in our study. 400 fish that were used to carry out the research work, pre enrichment was first done using peptone water, after which enrichment was done using brain heart infusion broth. It was then cultured on Ampicillin sheep blood agar which produced a grayish, circular, raised, beta hemolytic colonies. This shows the efficacy of these media in the isolation and confirmation of *Burkholderia Pseudomallei*.

According to [11], *Burkholderia pseudomallei* have been isolated from a wide range of animals including mammals, birds, reptiles, and fish which corroborates with our findings as we were able to isolate *Burkholderia pseudomallei* from fresh water fishes.

After gram staining and series of biochemical test such as oxidase test, catalase, citrate test, methyl red test, voges proskauer, motility, indole, hydrogen sulphide, and sugar fermentation tests such as inositol, glucose and mannitol. 40 isolates which were all gram negative, oxidase and catalase positive, motile, citrate positive, voges prokauer positive, produced hydrogen sulphide, glucose positive and very few negative, inositol negative and positive, mannitol positive and

few negative, urea positive and few negative were suspected to be *Burkholderia pseudomallei.*

Microbact

Aeromonas identified by the conventional biochemical test reaction were subjected to additional analytical profile index test using Microbact^{TM}24 GNB kit (Oxoid, England) which is a commercial biochemical kit in micro plate format for identifying Enterobacteriaeceae and Miscellaneous gram negative Bacilli. The organisms were identified based on pH change and substrate utilisations as established by published reference methodologies [19]. The kit has Microbact™ Computer Aided Identification Package that is consulted for the identification choices. The percentage figure shown against the organism name is the percentage share of the probability for that organism as a part of the total probabilities for all choices. It has been used by different researchers such as [20] for the identification of microorganisms. It is cheaper, and is easy and convenient to use, and is simplify and automate the identification of individual organisms. According to [4]. *Burkholderia pseudomallei* may be reliably identified using commercial lit systems for bacterial identification. And as such Microbact 24E system was used in this study which confirm 25 isolates as *Burkholderia pseudomallei.*

Distribution

Among all African countries, Nigeria is predicted to have by far the highest burden of melioidosis [9]. In sharp contrast to this potential disease load, the only report of melioidosis linked to Nigeria was documented in 2011, in which a case of a diabetic traveler was described who most likely acquired infection during a visit to the country [14].

[12] carried out to determine the effects of methanolic extract of bitter kola on bacterial isolates from sputum of patients with respiratory tract infections using one hundred and sixty patients, made up of 85 males and 75 females, attending chest clinics at Ekiti State University Teaching Hospital and General Hospital, both in Ado Ekiti, Nigeria. 5% of *Burkholderia* specie was isolated which is lower than the prevalence rate gotten in this study this might be due to the type of sample used in their study.

V. CONCLUSION

Our research was able to achieve its objectives, we were able to isolate *Burkholderia pseudomonallei* using Microbact^{TM} 24E identification kit with an overall prevalence of 6.25% and prevalence in FCT were Bwari area council is (8.73%), AMAC (5.34%) and Gwagwalada is 5.76%. The presences of *Burkholderia pseudomallei* might be through the ingestion. The existence of these zoonotic *Burkholderia pseudomallei* in fishes in FCT will imply potential biological chemical weapon therefore calls for establishment of *Burkholderia pseudomallei* laboratory in Federal Capital Territory for more researchers that will harness the organism. Detailed molecular studies should be conducted to expose more virulent strains of the organisms. Studies should also be conducted in horses and other organism to understand the epidemiology of the disease.

VI. RECOMMENDATION

Isolation of *Burkholderia pseudomallei* should be done in Federal Capital Territory (FCT) Abuja using higher molecular techniques such as the polymerase chain reaction (PCR) which is more sensitive.

Public health awareness and enlightenment of the dangers associated with *Burkholderia pseudomallei* in Nigeria is necessary.

Assistance is necessary from research centers in developed countries to enhance study of *Burkholderia pseudomallei* isolate in Nigeria.

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