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Antimicrobial Susceptibility Of Paenibacillus Species Isolated From Pig Farms In Ogun State.

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ABSTRACT

One hundred and thirty eight (138) pig faecal samples were collected and screened for the presence of paenebacillus sp. Isolates were classified into groups and 7 group of isolates had similar biochemical characteristics which were further identified using the 16SrRNA gene (27F and 1492R) as *Paenibacillus konsidensis*, *Paenibacillus massiliensis, Paenibacillus timonensis, Paenibacillus terrae, Paenibacillus phaseoli, Paenibacillus polymyxa, Paenibacillus amyloticus* for group 1-7 respesctively. The isolates were screened for their antimicrobial susceptibility using the broth microdilution technique and their resistance was confirmed using plasmid analysis. The Antibiotic susceptibility testing demonstrated Minimum Inhibitory Concentration (MIC) values of of 3.91- 62.5mg/ml for ciprofloxacin; tetracycline and clindamycin ranged had 31.25-125 mg/ml; while metronidazole, chloramphenicol and erythromycin showed MICs of >125.

Keywords: Paenibacillus, 16SrRNA, MIC, antimicrobial

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INTRODUCTION

Paenibacillus species are spore-forming rod-shaped facultative anaerobic gram positive bacteria. Species of *paenibacillus* have been detected in a variety of environments including fresh and salt water, soil, sewage, sediments, caves, humus, compost, rhizosphere, forage, insect larvae, food, plants and clinical samples (Padda et al., 2016a and b). Morphologically, colonies of *paenibacillus species* are very transparent with a milky-white color. The margin is entire and the surface is glossy and convex, with no noticeable odor (Slonczewski and Forster, 2017). These groups of organism are known to yield strong antimicrobial lipopeptides including antifungal, antibacterial, anticancer and antiviral enzymes (Cochrane and Vederas, 2016). Species of *Paenibacillus* are also involved in nitrogen fixation, phosphate solubilization and iron acquisition which promote plant growth (Grady et al, 2016; Padda et al., 2016a and b). The pathogenic characteristics of these isolates have been reported by de Graaf *et al*. (2006) and Garcia-Gonzales and Genersch (2013) where they attached bee hives causing the popular americal Foulbrood. Years later, Grady et al, (2016) also posits that some species of *Paenibacillus* are opportunistic pathogens in human especially immune-compromised individuals (Abrishami *et al.,* 2015). They are associated, but not shown to be directly involved in the causation of chronic kidney disease, sickle cell disease, premature birth, Whipple's disease, hydrocephalus, skin cancer, chronic interstitial nephropathy, and acute lymphoblastic leukemia (Nasu *et al*., 2003, Roux *et al*., 2004, 2008, and Ouyang *et al.,* 2008). These species ccan also be beneficial as they have been found useful in the production of industrial chemicals like enzymes, pesticides and biologically active metabolic products (Zamost *et al*., 1991; Lee *et al.,* 2012, 2016; Li *et al*., 2016).

So much work in Nigeria has been done on the detection of *Paenibacillus* species from the Rhizosphere of several plants as well as their application in Production of valuable products (Ogunmefun *et al*., 2015; Nwinyi and Amund, 2016). To the best of our Knowledge, this work is the first to report the isolation of *Paenibacillus* species in pig farms as well as their antimicrobial susceptibility in Nigeria. This will have great implications for public health especially individuals involved in Pig farming and pork consumption as well as those who desire to use the isolates as biorefineries.

MATERIALS AND METHODS

Study Site And Design

This study was carried out in two locations Otta and Oke-Aro pig farms in Ogun states of Nigeria. This state is selected because of its relatively high livestock activities, it has sizeable expanse of arable land and rich fertile soils that are good for the cultivation of a wide variety of food crops and animal production. Generally, livestock activities in the area are on the increase and there is a high dependence on livestock as a source of employment, revenue and milk and meat production. There are large-scale livestock production industries particularly of cattle, sheep and goats in these areas, mainly in semi-intensive farming systems in which animals are taken out to graze and then returned to their pens later in the evening, besides other small intensive and semi-intensive livestock farming operations that characterize most households. In addition, there is unregulated access to veterinary drugs; a farmer could decide to purchase and administer drugs without veterinary prescription and supervision.

Collection of Stool Sample

A total of 138 pig faecal samples (72 from Otta farms and 66 from Oke-Aro farms) were collected between January and February, 2017 in sterile universal stool containers. The samples were transported to microbiology laboratory of university of Lagos, within 2hrs for processing. Samples were not collected in any preservative. Prior to discarding, specimen were sterilized by autoclaving.

Culture And Isolation

Streak plate method was used to isolate anaerobes from pig stool sample. The media used in this study included, Columbia agar with 5% defibrinated sheep's blood (Oxoid, UK), non-selective media used were Fastidious Anaerobe Agar (FAA, Lab M), cooked meat broth (Oxoid, UK) and Mueller Hinton Agar (SRL, India). All samples were subjected to alcohol shock treatment by homogenizing the specimen with an equal volume of 95% ethanol at room temperature for 1 hr to destroy all vegetative cells leaving only spores. They were

subsequently dispensed into bottles containing Robertson's cooked meat Broth (CMB) for enrichment and the lids tightly covered.

The mixtures was then allowed to settle and a loopful of the deposit was aseptically streaked on Columbia agar for 48 hours, colonies after 48hrs of incubation were visible with the following characteristics, shiny, smooth with highly irregular forms, Colonies were then sub-cultured onto Fastidious Anaerobe Agar (FAA, Lab M) to obtain pure colonies in N_2(80%), H_2(10%) and CO_2 (10%), plates were incubated anaerobically at 37°C for 48hrs in an Anaerobic Jar (Biomerieux, France) in N_2(80%), H_2(10%) and CO_2 (10%). The surfaces of the plates were dried in the incubator at 30°C before use. Inoculated plates were incubated immediately under anaerobic conditions in an anaerobic jar (Biomerieux, France) in N_2(80%), H_2(10%) and CO_2 (10%) with an indicator (the rezusurin strip) to check anaerobiosis (disposable anaerobic indicator).

Media was reduced prior to inoculation by placing under anaerobic conditions for 24 h prior to use through the use of GasPak anaerobic systems. All plates showing no growth were further re-incubated for another 24 hours before being discarded. Control culture strain of *Pseudomonas aeruginosa* were plated alongside, this was to check anaerobiosis, because anaerobes grow well in anaerobic condition while *P. aeruginosa* which is a strict aerobe will not grow in the anaerobic jar. Pure cultures were then inoculated and stored into prepared cooked meat broth for morphological and biochemical characterization.

Antibiotic Susceptibility Testing: Broth Microdilution Method

The method was performed on commercially available 96-well broth microdilution plates for monitoring resistance of anaerobic and Gram-positive bacteria. Six antimicrobial agents were tested, including: erythromycin, tetracycline, metronidazole, chloramphenicol, clindamycin and ciprofloxacin. The isolated colonies were selected from 48 h anaerobic culture on Fastidious Anaerobe Agar (FAA, Lab M). The suspensions and inoculation of microdilution plates were carried out in an aerobic atmosphere, but the organisms were not exposed to air for more than 30 min. The procedure was carried out using reduced cation-adjusted Mueller Hinton broth for initial suspension which was transferred to a broth microdilution plate following the manufacturer's instructions. The plates were incubated at 35° C for 48 h in anaerobic conditions (GENbox anaerobic jar and GENboxAnaer generators; bioMe´rieux). The MIC end points were determined where no growth was observed, or, in cases where growth was observed in the last tested dilution, the results were interpreted as at or above the next twofold dilution.

Molecular Analysis

The DNA was extracted using the Jena Bioscience Bacteria DNA Preparation Kit (Germany), according to manufacturer's instruction and the 16SrRNA gene (27F and 1492R) were amplified using PCR. Agarose Gel Electrophoresis was used to separate the DNA into bands according to their molecular weight and DNA bands were visualized by ethidium bromide staining. PCR purified products were sequenced at Inqaba (South Africa) using Sanger sequencing and the corresponding sequences were identified at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Finally the Plasmid extraction was done using the mini prep TENS method as described by Zhou *et al*., (2016).

RESULTS AND DISCUSSION

The Antibiotic susceptibility testing demonstrated Minimum Inhibitory Concentration (MIC) values of of 3.91- 62.5mg/ml for ciprofloxacin; tetracycline and clindamycin ranged had 31.25-125 mg/ml; while metronidazole, chloramphenicol and erythromycin showed MICs of >125 mg/ml as shown in table 2. Figure 2 shows Plasmid analysis with the presence of plasmid on some of the *Panibacillus* species with a high molecular weight of 23,130 kbp. Seven different group of isolates were recovered by culturing and were represented by the biochemical profile that is presented in table 1. The identity of this microbe was determined by biochemical test and was further confirmed by molecular analysis using 16sRNA sequencing. Although, *Fontibacillus*, which is predominantly an environmental isolate (Jose *et al*., 2014; Lee *et al*., 2011) , was also identified, *Paenibacillus* was most frequent, species identified included; *Paenibacillus konsidensis*, *Paenibacillus massiliensis, Paenibacillus timonensis, Paenibacillus terrae* from Oke-Aro farm and *Paenibacillus phaseoli, Paenibacillus polymyxa, Paenibacillus amyloticus, Fontibacillus phaseoli* from Otta farm with a range of 95-99% identity. There was no report of other pathogenic fecal organisms commonly found in pig feces in our data which is in contrast

with a report by Baer and his colleagues (2013), who reported the prescence of *salmonella* and *campylobacter in* pig feces, however, this may be due to the fact that most *Paenibacillus* strains have been reported to produce a variety of antimicrobial substance Lee *et al*., (2012) and as such inhibits other pathogens.

Previous studies have reported the isolation of different strains of this organism in patients undergoing haemodialysis, prosthetic joint infection, bacteremia in cerebral infection, Carcinoma intestinal nephropathy and leukemia (Nasu *et al*., 2003, Roux *et al*., 2004, Ko *et al*., 2008, and Ouyang *et al.,* 2008).Although they are also relevant to animals, plants and the environment (Grady *et al*., 2016) the pathogenicity of *paenibacillus spp* cannot be ruled out as it has also been reported as the causative agent of American foulbroad, a lethal disease of honeybees (Garcia-Gonzales and Genersch, 2013) and snail disease a causative agent of Schistosomiasis (Grady *et al*., 2016, Duval *et al*., 2015). P*aenibacillus spp* being an opportunistic human pathogens, several of which have been isolated from humans globally (Grady *et al*., 2016) has been associated with diseases such as chronic kidney disease (Padhi *et al*., 2013), premature birth (Deleon and Welliver, 2016), whipple disease (Roux *et al*., 2008), skin cancer, chronic interstitial nephropathy and acute lymphoblastic leukemia (Roux *et al*., 2004).

Although *peanibacillus* has been implicated in all these infections, no study has linked these organism in gastrointestinal infections in animals and humans, hence the concern for the result of this study which seem to have implicated *peanibacillus* in diarrhoeic stool from pigs, prior to this study many of the piglets suffered diarrhoeic infection of which over 75% of them died and the cause was not determined due to inadequacy/ difficulty in accessing laboratory materials for the analysis of anaerobic infection in animal farms in Nigeria. Hence further studies is required to determine the role of *peanibacillus* in gastrointestinal infections in pigs.

The antibiotics drug susceptibility testing demonstrated susceptibility in ciprofloxacin with MIC variability of 3.91- 62.5mg/ml, lower susceptibility was also seen in tetracycline and clindamycin with MIC range from 31.25-125 mg/ml while metronidazole, chloraphenicol and erythromycin showed high multiple drug resistance with MICs of >125 mg/ml, this drug resistance identified supports a study by Grady and his associates (2016), who demonstrated a variety of strain-dependent drug resistances to norfloxacin, clindamycin and ampicillin in *Paenibacillus* isolated from humans.

Plasmid analysis showed that the multiple resistance portrayed by some of the *paenibacillus species* in this study were plasmid mediated, this report is in line with a study by Soundarapandian and associates (2012), who reported plasmid-borne drug resistant *paenibacillus species* isolated from crab, although their plasmid molecular weight was about 1300kbp which is much less than the findings in this study which was calculated to be 23130kbp. Antibiotic drug resistance identified in this study points out possible transfer of antibiotic resistance from pig faeces to fertile soil which may occur through production and distribution of pig manure in agriculture as reported in a study by Faldynova and colleagues (2013), that animal manure is a reservoir for antibiotic residues, antibiotic resistant bacteria, human pathogens and antibiotic resistant genes, hence when this manure is applied to the soil, these antibiotic resistant genes may be transferred to other soil pathogens including human pathogens.

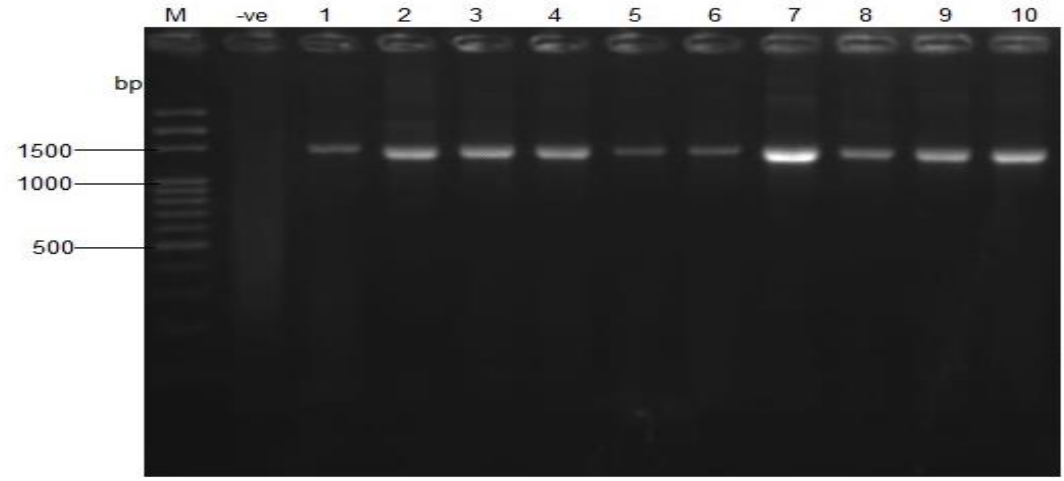

Figure 1: Agarose gel electrophoresis (1.5%) showing lane 1-10 PCR amplified products of 1500bp. –ve is negative control of PCR mix without DNA and M is a 100 base pair ladder.

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Table 1: Groups of isolates with similar biochemical activities

	NUMBER OF ISOLATES IN SAME GROUP WITH SIMILAR TEST ACTIVITIES						
BIOCHEMICAL TEST	**7 Group A**	**9 Group B**	**4 Group C**	**1 Group D**	**5 Group E**	**8 Group F**	**2 Group G**
motility	+	+	+	+	+	+	+
anaerobiosis	+	+	+	+	+	+	+
Starch hydrolysis	+	-	+	+	+	+	+
catalase	-	-	+		+	+	+
Oxidase activity	-	-	-	+	-	-	-
Hydrogen sulphide production	-	ND	ND	ND	-	+	ND
Vogues proskeur	-	-	-		-	+	ND
indole	-	ND	ND	ND	-	-	ND
Spore formation	+	+	+	+	+	+	+
Nitrate reduction	+	-	+	+	+	-	+
Acid production test: Glycerol	-	-	+	+	+	+	+
Ribose	+	-	+	-	+	+	+
Xylose	-	+	+	-	-	+	+
Inulin	-	+	-	-	+	+	+
Sucrose	+	+	+	-	+	+	+
Mannose	+	-	+	-	+	+	+
Mannitol	-	+	+	-	+	+	+
Glycogen	+	-	+	+	+		+
Gluconate	+	-	-	+	-	ND	+
Arabinose	-	-	-	-	+	-	-

KEY: + positive reaction, - negative reaction, and ND not determined

Table 2: Minimum inhibitory concentration (antibiotics susceptibility) using Broth dilution method.

ISOLATES	**ANTIBITOTICS**					
	CIP mg	**TET mg**	**CDA mg**	**MTZ mg**	**CHL mg**	**ERY mg**
Group A	3.91	31.25	>125	>125	>125	>125
Group B	31.25	62.50	>125	>125	>125	>125
Group C	62.50	62.50	>125	>125	>125	>125
Group D	62.50	>125	>125	>125	>125	>125
Group E	62.50	62.50	31.25	>125	>125	>125
Group F	62.50	>125	>125	>125	>125	>125
Group G	62.50	62.50	62.50	>125	>125	>125

key: CIP Ciprofloxacin (200mg)
MTZ Metronidazole (1000mg)
TET Tetracycline (250mg)
CDAClindamicin (150mg)
CHL Chloramphenicol (500mg)
ERY Erythromicin (125mg)

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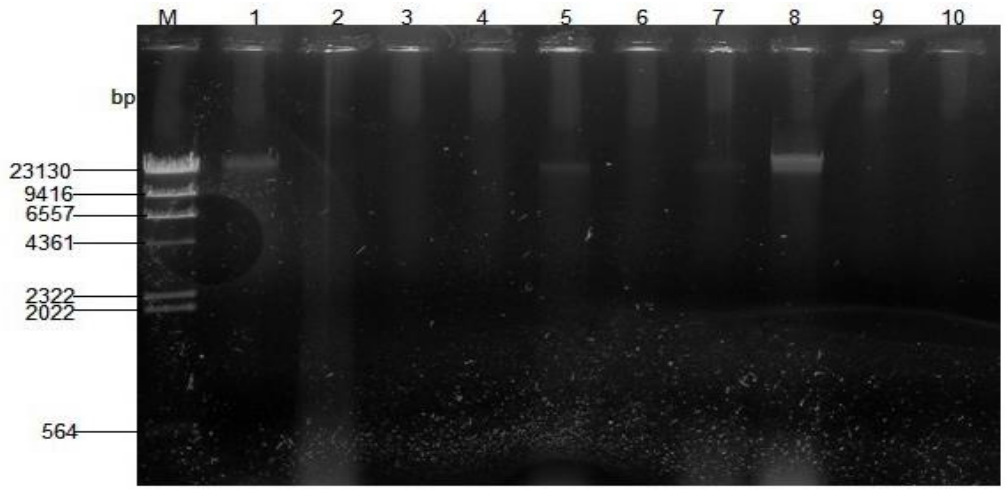

Figure 2: Agarose gel electrophoresis showing the presence of plasmid in lane 1, 5, 7 and 8 with Lane M as Lamda Hind III molecular weight marker.

CONCLUSION AND RECOMMENDATION

Paenibacillus spp which was commonly considered soil-related organisms are increasingly isolated from hospitalized patients and appear sufficiently equipped of virulence properties. In this study, they were implicated in gastroenteritis in pigs and this is worrisome as this is the first time to the best of our knowledge that this organism are been isolated from diarrhoeic pig faeces in Nigeria, also it is of particular concern that these organisms displayed multiple-drug resistance, hence its of utmost importance that the isolation of bacteria belonging to the *Paenibacillus* genera should not be disregarded and their identification should be performed, particularly when isolated from samples derived from food animals. This study showed that Ciprofloxacin and tetracycline were effective in inhibiting the growth of *Paenibacillus* and therefore could serve as good antibiotics in treatment of *Paenibacillus* related infections, it also showed multiple drug resistance to common antibiotics such as metronidazole, clindamycin, erythromycin and chloramphenicol in this regards such multi-drug resistance factors in *Paenibacillus* may add intimidation of transmission of resistance to common soil originating pathogens. Hence it is important that antibiotics are only used when absolutely necessary, to avoid occurrence of drug resistance in Nigerian farms**.** Moreover it should be noted that *Paenibacillus*, being an atypical pathogen of human and animals, should never have been exposed to an antibiotic as such except those produced in soil micro-flora and also risk reduction strategies should be encouraged throughout the food chain, these stratagies includes increased hygiene at both slaughter and meat processing and continued implementation of HACCP systems to prevent future outbreaksfrom occurring.

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