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SEARCH FOR THE OCCURRENCE OF *CLOSTRIDIUM DIFFICILE* AND *CLOSTRIDIUM PERFRINGENS* IN PIGS WITHIN ZARIA AND ENVIRONS, IN KADUNA STATE, NIGERIA

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ABSTRACT

Animals have been known to be the main study subjects when investigating the epidemiology of zoonotic Gram-positive *Clostridium difficile* and *Clostridium perfringens*. This cross-sectional study was aimed at determining the occurrence of *C. difficile* and *C. perfringens* in pigs as well as the associated risk factors within Zaria and environs, in Kaduna State of Nigeria. A pre-sampling survey led to the selection of Shika, Samaru and Ungwan Mangu in the pig farming communities of Zaria and environs in Kaduna North as the study sites. Rectal swabs from 132 pigs were obtained and anaerobically cultured in fluid thioglycolate and further grown on reinforced clostridia agar. The colonies obtained were sub-cultured in *Clostridium difficile* moxalactam norfloxacin agar and reinforced clostridia agar containing egg yolk tellurite. *C. difficile* was not detected. However, *C. perfringens* was detected at a prevalence of 16.7 % (22/132). Isolates were tested for their susceptibility to 13 antimicrobials. Only 1 isolate (4.55 %) demonstrated susceptibility to vancomycin, gentamicin, chloramphenicol and erythromycin. Of the bivariate analyses of the risk factors studied, only the type of piggery and pig management were statistically significant ($P < 0.05$) for *C. perfringens*. Therefore, it should be recognized that there is a need for pig farmers to be enlightened about this pathogen and its prevention through good management practices and hygiene.

Key words: antibiotic susceptibility; *Clostridium difficile*; *Clostridium perfringens*; pig management

INTRODUCTION

Clostridium difficile (now reclassified as *Clostridioides difficile* [23, 32] and *Clostridium perfringens* are Gram positive, anaerobic spore-forming bacteria [4, 47]. They are among the most common causes of broad spectrum antibiotic associated diarrhoea (AAD) in humans and other animals [33, 39, 50]. The transmission of both *Clostridium* spp. among most animal species is mostly via faeco-oral and/or from the environment. The transmission is primarily due to their characteristics clostridia spores which

can overcome the unhospitable acidic condition of the gastrointestinal tract [29, 46].

After gaining passage to the large intestine, under proper conditions, clostridia organisms are able to germinate and colonize the large intestine. *C. difficile* produces large glucosylating exotoxins toxin A (TcdA) and the enterotoxin toxin B (TcdB), which results in the characteristic pathology of *C. difficile* infections. These toxins are considered the major virulent factors associated with *C. difficile* disease [9, 15, 26]. Some strains of *C. difficile* produce an adenosine diphosphate ribosylating binary toxin; however, the role of this toxin in the pathogenesis of the disease associated with *C. difficile* is yet to be elucidated [15, 35].

C. perfringens is classified into seven strains: A, B, C, D, E, F and G [22, 41]. It has been reported that the clinical signs of clostridial diarrhoea in pigs are similar to those of several other enteric diseases [38] caused by enterotoxigenic *E. coli* [7, 10], *Cryptosporidium* spp. [24], *Salmonella enterica* [18] and *Staphylococci* spp. [27] making it difficult to determine its true prevalence in pigs [11]. The absence of routine clinical diagnoses for clostridial spp. diarrheal cases results in little attention being given to *Clostridium difficile* and *Clostridium perfringens* infections in pigs within Zaria and environs, in Kaduna State of Nigeria. This research was stimulated in order to provide a baseline data on the occurrence, antimicrobial susceptibility of *C. difficile* and *C. perfringens* in pigs and their associated risk factors within Zaria and environs, of Kaduna State, Nigeria.

MATERIALS AND METHODS

Study design

A cross sectional study design was used in this research from April—August, 2018. The snowball sampling technique (majority of residents are of Islamic faith) was used to identify owners with pigs in the settlements of Shika, Samaru and Ungwan Mangu. These three settlements were selected based on the presence and availability of pigs where faecal samples could be collected from pigs and questionnaires administered to the farm's owners or attendants.

This study was carried out in Zaria, in Kaduna state of Nigeria, which is positioned between latitude 11° 07"N and 11°12' N. and longitude 07°41'E. Zaria is in the centre of northern Nigeria and located on a plateau 2200 feet above sea level.

Zaria is characterized by a tropical climate, a monthly mean temperature ranging from 13.8—36.70 °C and an annual rainfall of 1092.8 mm. Dry season farming is the second most prevalent agricultural activity in Zaria with vegetables being the common produce, but in some cases fruits are sandwiched among the cereal crops [1]. It has an estimated population of 408,198 people (NPC, 2006). Animals being raised are mostly ruminants [3], although the pig population in Kaduna State has been estimated to be 249,651 [36] even though no recent census has been conducted.

Sample size determination

The sample size was determined using the formula by Thrushfield [44] at 95% confidence interval. Subsequently, a prevalence of 8.6% by Norman et al. [31] and a sample size of 121 was obtained. Later, 9.09% of 121 was added to reduce the sampling error and accommodate additional samples, thereby increasing the sample size to 132.

Isolation and culturing

Rectal swabs (n = 132) were cultured in fluid thioglycollate (Merck, KGaA, Germany) which were incubated aerobically at 37 °C for 24 hrs. The cultures were streaked onto reinforced clostridia agar and incubated anaerobically. The suspected colonies were then sub-cultured onto two media, first *Clostridium difficile* Agar Base (Oxoid, CM0601; Oxoid Ltd., Hampshire, United Kingdom) containing Moxalactam, Norfloxacin and Cysteine hydrochloride as selective supplements (CDMN, SR0173; Oxoid Ltd., Hampshire, United Kingdom) with 7% v/v horse blood and reinforced clostridial agar medium containing egg yolk tellurite. The streaked plates were incubated anaerobically using an anaerobic jar incorporated with a gas generating kit (Thermoscientific, AnaeroGenTM 3.5L, AN0035A, Oxoid Ltd, Wade Road, Hants, UK) at 37 °C for 48—72 hrs according to a modified method of Tizhe et al [45]. The suspected isolates of *C. difficile* were viewed for grey white colonies on CDMN agar while those grown on blood agar were viewed under ultraviolet light for the characteristic morphology of *C. difficile* with yellow green fluorescence while suspected isolates of *C. perfringens* were viewed for black colonies on reinforced clostridial agar while those grown on blood agar were viewed for yellow grey colonies and a double zone of haemolysis on blood agar.

Biochemical typing of *Clostridium* isolates

Suspected isolates were subjected to the following conventional array of biochemicals: the nitrate reduction test, lecithinase test, sugars (glucose, lactose, mannitol, inositol, dulcitol, arabinose, sucrose and rhamnose), L-arginine test, catalase test, triple sugar iron test, urease test, indole test and methyl red Voges Proskaeuer test [5, 17, 20].

Antibiotic sensitivity testing

The susceptibility testing was performed using the Kirby-Bauer disk diffusion method [6]. The antibiotics used were those commonly used by farmers: vancomycin (30 µg), gentamicin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg), streptomycin (10 µg), oxacillin (1 µg), clindamycin (2 µg), cefoxitin (30 µg), penicillin (10 µg), sulphamethazole-trimethoprim (25 µg), amoxycillin-clavulanic acid (30 µg) and imipenem (10 µg). The zones of inhibition were measured and compared with the Clinical and Laboratory Standards Institute [13] and the European Committee on Antibiotic Susceptibility Testing [16] guidelines.

Questionnaire administration

A structured questionnaire (73) was administered to the owners of the pig farms that were sampled to obtain information on pig management, attitude and practices as it related to the risk of possible acquisition of *C. difficile* and *C. perfringens* infections in pigs.

Data analyses

The statistical Package for Social Sciences (SPSS VERSION 21.0) was used to analyse the data obtained. The Fisher's exact test was used to show associations between *C. perfringens* and risk factors. The risk factors were assessed using regression and odd ratio at 95% confidence interval. The absence of a risk factor was considered as a reference category for odds ratio. The values of $P < 0.05$ were considered significant. The data obtained were presented in the subsequent tables.

RESULTS

There was no *C. difficile* isolated from pigs but instead, only *C. perfringens* were isolated. Table 1 shows the number of animals that were positive for *C. perfringens*, from Shikah and Samaru. None were positive from Ungwan Mangu, while out of all of the pigs sampled (132), only 22 (16.7%) pigs were positive for *C. perfringens*.

As to the antibiotic susceptibility test carried out for the various isolates, only 1 isolate (4.6%) was susceptible. All of the other isolates were resistant to: vancomycin, gentamicin, chloramphenicol and erythromycin while there was 100% resistance to ciprofloxacin, streptomycin, oxacillin, clindamycin, cefoxitin, penicillin, imipenem, sulphamethazole-trimethoprim and amoxycillin-clavulanic acid (Table 2).

Possible environmental risk factors associated with the presence of *C. perfringens* infections

Based on the analyses of the responses to the questions on the type of piggery in relation to the presence of *C. perfringens*, 3 (4.2%) of the farmers having the backyard type of piggery had the *C. perfringens* in the faeces of their pigs, while 68 (95.2%) had an absence of *C. perfringens* in the faeces of their pigs, and 2 (100%) of those with established farms had an absence of *C. perfringens* from the rectal swabs collected from their pigs. Also, the response to questions on pig management in relation to *C. perfringens* showed that the pigs which were reared under intensive, semi-intensive and extensive to be 14.3%, 1.6% and 100% had the presence of *C. perfringens* while there was an absence of *C. perfringens* in pigs of those who were raised under intensive 6 (85.7 %), semi-intensive 63 (98.4%) methods (Table 3).

Table 1. Distribution of *C. perfringens* isolates based on the location and gender in Zaria and environs

Sampled area	Sex	No. of pigs sampled	No. of pigs positive (%)
Shikah	Male	30	13 (43.3)
	Female	20	6 (30.0)
	Total	50	19 (38.0)
Samaru	Male	42	2 (4.7)
	Female	20	1 (5.0)
	Total	62	3 (4.8)
U/Mangu	Male	16	0.0
	Female	4	0.0
	Total	20	0.0
Total		**132**	**22 (16.7)**

Table 2. Susceptibility of *C. perfringens* isolates from pigs to routinely used antimicrobials in Zaria and environs

Antimicrobial agent (concentration)	Susceptible (%)	Intermediate (%)	Resistant (%)
Vancomycin (30 µg)	1 (4.5)	0 (0.0)	21 (95.45)
Gentamicin (30 µg)	1 (4.55)	0 (0.0)	21 (95.45)
Chloramphenicol (30 µg)	1 (4.55)	0 (0.0)	21 (95.45)
Erythromycin (15 µg)	1 (4.55)	0 (0.0)	21 (95.45)
Ciprofloxacin (5 µg)	0 (0.0)	0 (0.0)	22 (100)
Streptomycin (10 µg)	0 (0.0)	0 (0.0)	22 (100)
Oxacillin (1 µg)	0 (0.0)	0 (0.0)	22 (100)
Clindamycin (2 µg)	0 (0.0)	0 (0.0)	22 (100)
Cefoxitin (30 µg)	0 (0.0)	0 (0.0)	22 (100)
Penicillin (10 µg)	0 (0.0)	0 (0.0)	22 (100)
Imipenem (10 µg)	0 (0.0)	0 (0.0)	22 (100)
Sulphamethazole-trimethoprim (25 µg)	0 (0.0)	0 (0.0)	22 (100)
Amoxycillin-clavulanic acid (30 µg)	0 (0.0)	0 (0.0)	22 (100)

Table 3. Response of pig owners to questions on the type of piggery and pig management in relation to presence/absence of *C. perfringens* in Zaria and environs

Variable	Present (%)	Absent (%)	Odd ratio	C. I value	P value
Type of piggery backyard	3 (4.2)	68 (95.2)	0.44	0.20—0.95	0.001
Established pig management system	0 (0.0)	2 (100)	REF		
Intensive	1 (14.3)	6 (85.7)	0.47	0.08—1.44	0.001
Semi-intensive	1 (1.6)	63 (98.4)	1	0.14—13.38	
Extensive	1 (100)	0 (0.0)	REF		

C.I—95 % confidence interval. Fisher's exact test; REF—Reference category

Table 4. Response of pig owners to questions on the keeping of other animals and allowing other boars to service their sows in relation to presence/absence of *C. perfringens* in Zaria and environs

Variable	Present (%)	Absent (%)	Odd ratio	C. I value	P value
Those who keep animals other than pigs					
Yes	2 (4.4)	43 (95.6)	1.2	0.10—14.0	1
No	1 (3.7)	26 (96.3)	REF		
Those who carry their pigs to be serviced by other boars outside the farm					
Yes	3 (4.8)	59 (95.2)	0.95	0.90—1.0	0.68
No	0 (0.0)	11 (100)	REF		

CI—95 % confidence interval (Fishers exact test); REF—Reference category

Table 5. Type of piggery and management systems of pigs within Zaria and environs

Variables	Frequency	Percentage (%)
Type of piggery		
Backyard	71	97.3
Established	2	2.7
Pig management		
Intensive	7	9.6
Semi-intensive	65	89
Extensive	1	1.4

Respondents who noted yes (4.4 %) and no (3.7 %) to questions on keeping animals other than pigs had *C. perfringens* from the rectal swabs collected from the pigs even though there was no statistical significance (OR-1.2 p value 1.0), while respondents who said yes (4.8 %) to questions on carrying their pigs to be service by other pigs outside the piggery had *C. perfringens*, while 95.2 % had an absence of *C. perfringens* even though no statistical significant association was evident (Table 4).

Table 5 shows the frequency of pig holders within Zaria who had the backyard (97.3 %) and established (2.7 %) type of piggery, as to pig management; 9.6 % practiced the intensive pig management, while 89 % practiced the semi-intensive management and 1.4 % practiced the extensive management.

DISCUSSION

In this study, the isolation of *C. difficile* was attempted, but none was recovered. It has been shown that the prevalence of *C. difficile* carriage in swine drops dramatically with age [28, 49] and perhaps that may have been the reason for the absence of *C. difficile* in this study, as most of the pigs sampled were adults of reproductive age. However, *C. perfringens* was isolated successfully. The shedding of these two organisms in faeces constitutes a risk factor to piglets. Ferreira et al. [19] established a high prevalence (58.8 %) of *C. perfringens* from the 90 pigs sampled. This difference in the prevalence between *C. difficile* and *C. perfringens* may have been due to different practices employed by the pig owners or genetic factors of the particular breed of pigs. *Clostridia* spp. are usually the first colonizers of the piglet gastrointestinal tract; thereby predisposing the piglets to infection which might later lead to diarrhoea [42].

Antibiotics are used as therapeutics, growth promoter and as a prophylaxis [14]. The 22 isolates in this study were subjected to 13 antibiotics, of which only one isolate was susceptible to gentamicin, chloramphenicol and erythromycin. Perhaps a different strain of *C. perfringens* was present among the isolates. Antimicrobial resistance among anaerobes has consistently been on the increase in the past 3 decades and the susceptibility of anaerobic bacteria to antimicrobial agents has become less predictable especially among anaerobes, such as *Clostridium* spp., that were previously very susceptible [8]. This increase resistance makes

the choice of appropriate empirical therapy even more difficult in a developing country like Nigeria since a lot of farmers have no knowledge of *C. perfringens*, but tend to attribute diarrhoea caused by this bacterium to other bacteria and parasites.

Our work is at variance with the reports of van den Bogaard et al. [48] and Ngamwongsatit et al. [30] who reported the resistance of *C. perfringens* isolates from pigs to chloramphenicol and erythromycin. The reason for the susceptibility seen in our study might be due to the fact that the resistance had not yet developed in this isolate. Also, in a recent study in Lagos, Nigeria, *C. perfringens* isolated from food and human faecal specimens were resistant to erythromycin, in which the authors reported that some of the isolates might have originated and transmitted through faecal contamination of food [12].

As to the penicillin and clindamycin resistance, it has been reported by the CLSI [13] that some isolates of *C. perfringens* are resistant to these two antibiotics which was observed in our study, even though pig farmers use most of the antibiotics for empirical therapy against other known pathogens that may mask or confound the clinical signs of *C. perfringens* with rare visits to the veterinarian for confirmation of the causal organism (based on personal communication with most of the farmers). Antimicrobial resistance reported from livestock are mostly of anthropogenic origin [2]. A lot of resistance studies to a wide range of isolates tested against antimicrobials belonging to different classes of antibiotics in Nigeria which shows that almost in all instances, resistant pathogens were recovered from livestock (pigs, goats, cattle, sheep, chickens and camels). Also, the most available information on resistance in Nigeria are products of "individuals" effort studies [2], which perhaps is the reason why most researchers cannot go further to find out the sources of resistance. Reports of resistance to amoxycillin-clavulanic acid, vancomycin, imipenem, penicillin, chloramphenicol and ceftriazone (same group with cefoxitin) antibiotics have also been reported in isolates of *C. perfringens* in pigs from Thailand, Canada and Brazil [37, 40, 43].

Food producing animals are linked to humans through the food chain and shared environment. The possibility exist that antimicrobials residues will always be present in meats [21, 25, 34] of these pigs at the time of sale and consumption, and that is why Chukwu et al. [12] said that "there is need for periodic antimicrobial susceptibility testing and surveillance to detect geographic and temporal changes in the resistance trends of *C. perfringens*".

From our study, there were more backyard piggeries than established farms, and this might have been due to the fact that pig farmers need to keep a close watch over their pigs. Also, most of the pig owners might not be rich enough to own landed properties and the funds to run an establish farm. Most of these piggeries were located close to toilets (pit toilets) and with the bathroom drainages passing close to the piggeries partially serving as water for the pigs. This may serve as reason why pigs were being infected with *C. perfringens*. While sampling, it was observed that some farmers threw vegetables along these drainages and the pigs consumed these vegetables like corchorus stocks (ayoyo) and cabbage leaves. Perhaps this might be the reason for the strong association between the type of piggery and *C. perfringens*. Chukwu et al. [12] reported the presence of *C. perfringens* in cabbage in Lagos state of Nigeria and also, *C. perfringens* has been reported in water [42]. During the course of sampling it was discovered that most of the piggeries were not cemented; perhaps, another reason for the presence of *C. perfringens*, since one of their principal habitat is the soil [25].

Pig management has always been an issue in areas where pigs are raised because of the damage they cause to crops in the rainy season. One of the sampled areas that had the highest prevalence of *C. perfringens* did keep their pigs under strict intensive management. However, during the course of sampling it was observe that the association between the pigs and other animals in the environment was not different from other pigs that were being manage under the semi-intensive and extensive system. As a result, this could have contributed to the prevalence of *C. perfringens* through the faeces of the other animals that associate with the pigs. A lot of pig farmers practiced the semi-intensive management, in which the pigs were allowed to scavenge for food. These pigs returned in the evening and were given watery cereal bran. Prior knowledge of where the animals had been was unknown to the owners. This act of scavenging and consumption of contaminated feed and faeces may result in *C. perfringens* infections observed in this study, even though $P<160.05$, but the strength of the association between pig management and *C. perfringens* was weak.

CONCLUSIONS

C. difficile was not isolated but *C. perfringens* was found with a prevalence of 16.7 %. Most of these isolates were resistant to the antibiotics tested. Only 1 isolate (4.55 %) was susceptible to vancomycin, erythromycin, gentamycin and chloramphenicol. The environmental risk factors associated with *C. perfringens* included the type of piggery and pig management. The pre-dominance of smallholder/family pig farming has made it difficult to focus attention on the epidemiology of clostridial diseases in the pig industry here in Zaria and environs, Kaduna State, Nigeria.

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