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ISOLATION METHODS FOR MOLECULAR DETECTION AND ANTIBIOTIC RESISTANCE PATTERN OF *CAMPYLOBACTER SPP* IN LAYER CHICKENS

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ABSTRACT

This study was conducted to compare two culture methods for the isolation of *Campylobacter* spp from commercial layer chickens and subsequently confirmed by Polymerase Chain Reaction assays (PCR). Furthermore, the antimicrobial resistance profiles of PCR positive *Campylobacter* isolates were determined.Cloacal swab samples (550) from chickens randomly selected from five poultry farms in the four geographical zones in Ogun State were cultured for *Campylobacter* using modified charcoal Cefoperazone deoxycholate agar (MCCDA) and an improved culture method involving Preston broth pre-enrichment and subsequent subculture on Mueller Hinton agar with *Campylobacter* growth supplements. Putative isolates were later confirmed by PCR assay and sequencing analysis.Other isolates that grew on MCCDA and confirmed by sequencing analysis are *Enterococcus faecalis*, *Escherichis coli*, *Comamonas kerstli* and *Pseudomonas aeroginusa* . The antibiotic resistant profile of all the isolates were evaluated genotypically for resistance genes to tetracyclines (*tetO*), multiclasses (*cmeB*), aminoglycosides (*aphA-3-1*) and β-lactams (Bla_{oxa-61}) using multiplex PCR (mPCR), and phenotypically for chlortetracycline, tylosin, streptomycin, ciprofloxacin and erythromycin resistance by microbroth dilution method which correspond to the antibiotic resistance genes. The apparent prevalence of *Campylobacter* was 16.8% by MCCDA while none of the isolates was positive to PCR. Meanwhile, prevalence rate of 26% was obtained using Preston broth pre-enrichment and Mueller Hinton agar with *Campylobacter* growth supplements, of which 11/50 (22%) of the isolates was confirmed positive by PCR. Genotypic characterization of PCR positive isolates showed 10/11(90%) were *C. coli,* 1/11(10%) other *Campylobacter species* and 0% C. *jejuni.* All the isolates carried both *tetO* and *cmeB* resistant genes. The results of minimum inhibitory concentration presented all PCR positive isolates had resistance of 10/10(100%), 9/10(90%), 6/10(60%), 9/10(90%), and 8/10(80%) to tetracycline, ciprofloxacin, erythromycin, spectinomycin and tylosin respectively. In addition, all isolates carried multiple resistance to most antibiotics tested which are commonly used in poultry practice in Nigeria. *Campylo-*

bacter spp in the study areas showed diverse genotypic characteristics, and gene mediated multidrug resistance.

Keywords: antibiotic resistance, *campylobacter,* layer chickens, polymerase chain reaction, prevalence

INTRODUCTION

Campylobacter species are Gram-negative, non-spore forming micro-aerophilic or anaerobic, mainly spiral-shaped bacteria. They are mostly recognized as a cause of human food borne gastroenteritis worldwide [1, 2, 3].

Poultry and poultry products have been shown to be important sources of human Campylobacteriosis and play important roles in disease transmission [4]. Public awareness of *Campylobacters* as human pathogens has been relatively limited compared to other enteric bacteria in animals. *Campylobacters* have been implicated in diseases such as diarrhoea in cattle and septic abortion in both cattle and sheep [5]. Findings showed that prevalence of Campylobacters in humans and animals ranges from 3% to 98% [6, 7]. Due to lengthy analytical processing time associated with conventional culture methods for isolation and biochemical characterization of the *Campylobacter spp,* several attempts have been made to use molecular based techniques (e.g. Polymerase Chain Reaction (PCR) as a definitive identification and characterization method for bacteria. PCR is rapid, has high discriminatory power, and used in many developed countries [8]. However molecular techniques have not been so much pragmatic in Nigeria for diagnosis as well as epidemiological studies.

Although the use of antibiotics is not usually indicated in the treatment of non-complicated Campylobacteriosis, severe systemic and or chronic infection may call for antibiotic therapy. There has been reports of increase in antibiotic resistance of *Campylobacter species* to some commonly used antibiotics like fluoroquinolones and macrolides [3], and antimicrobial use has been shown to correlate with the emergence of resistant strains [9]. It is therefore needful to have a basic understanding of the association of C*ampylobacter* with layer chickens, especially the zoonotic *C jejuni* and *C. coli*; so that preventive strategies can be developed to reduce its occurrence and disease potential. Also there is need to adopt a sensitive isolation method for prompt and accurate diagnosis of infection as well as determine the antibiotic sensitivity profile of Campylobacter isolates

MATERIALS AND METHODS

Study Location

The study was carried out in Ogun State, South Western Nigeria and Ohio State, United States of America. Ogun State is made of 20 Local Government Areas divided into four geographical zones namely Egba, Ijebu, Yewa and Remo. The State between latitude 6.2^{0}N and 7.8^{0}N and longitude 3.0^0 and 5.0^{0}E at an elevation of 169 feet above sea level with an area of 16,762 square kilometers and a human population of 4,054,272. Poultry production is the main livestock farming in the state with a population representing, 40% of 150.7 million of the total poultry population in Nigeria. Chickens (both local and exotic) are predominantly reared intensively, semi-intensively and extensively in Ogun State

Ohio State is in the Midwestern United States of America. It lies in latitude 38^{0}24'N

to 41°59'N and longitude 80°31'W to 84°49'W at an elevation of 850 feet above sea level with a total area of 116, square kilometers and a human population of 11,594,163.

Study design, farm recruitment and sample collections

A cross sectional survey design was conducted to investigate the prevalence and antibiotic resistance profile of *Campylobacter species in* faeces of commercial layer chickens using culture and PCR methods. For farm recruitment, purposive sampling was conducted. For the first phase of the study, four major cities from the four (4) geographical zones: Ijebu (Ijebu-Ode), Egba (Abeokuta), Remo (Sagamu) and Yewa (Igbesa) were sampled based on the high concentrations of commercial poultry farm populations in these locations. Subsequently, five (5) commercial poultry farms from each of the four major cities were enrolled for the study (20 farms). A systematic random sampling method was used to choose 25 birds per farm resulting in a total of 500 cloacal swabs. For the second phase, fifty (50) laying chickens were sampled from one of previously selected poultry farms in the first phase. All samples were analyzed both at the Veterinary Microbiology and Public Health laboratory, Federal University of Agriculture, Abeokuta. Ogun State, Nigeria, and the Food Animal Health Research Program (FARHP), the Ohio State University Agriculture Research Development Centre, United States of America (USA).

Culture methods for identification of Campylobacter

The samples collected were placed in sterile Amies transport medium prepared in bijou bottles (w/v; 5mls), and kept on ice for transportation to the Laboratory. The samples were analyzed within 8 hours post collection at Veterinary Microbiology and Public Health laboratory, Federal University of Agriculture, Abeokuta. Ogun State, Nigeria. Bacterial isolation and biochemical identification were carried out according to [10]. Briefly, individual swabs were transferred to 7 ml buffered peptone water and incubated micro-aerobically at 42 °C for 48 hours in an anaerobic jar containing a microaerophilic generating sachet (Campygen®, Oxoid). Thereafter, a loopful of culture broth was inoculated into *Campylobacter* selective medium consisting of modified charcoal cefoperazone deoxycholate agar (MCCDA, Oxoid® CM73) supplemented with cefoperazone and amphotericin B (Oxoid®, SR155). The inoculated plates were incubated at 42 °C for 48 hours in an anaerobic jar containing microaerophilic generating sachet (Campygen®).

Subsequently, five colonies with colonial morphology consistent with *Campylobacter species* were purified on blood agar and incubated for 24 hours at 42 °C. Motility [11], catalase and oxidase [12], gram staining and hippurate hydrolysis tests [13] were performed on purified isolates. Isolates were stored in glycerol (v/v, 80%) at – 80°C until transportation to the laboratory of Food Animal Health Research Program (FARHP), the Ohio State University Agriculture Research Development Centre, United States of America (USA) for further molecular characterization. Prior to shipment, glycerol stocks were subcultured on MCCDA plates and incubated as earlier described. Thereafter, purified colonies were suspended in Wilkins Chalgrin transport medium® (Oxoid) and incubated for 48hours at 42°C under microaerophilic condition. These were then packed with dry ice in cooler and shipped to the USA.

On the other hand, samples collected for phase 2 of study (50 cloacal swabs) were transferred into Bijou bottles containing 6 mls of maximum recovery diluents (MRD), and later analyzed using an improved protocol of bacterial isolation method Briefly, 1ml of the samples were transferred into test tubes containing 7 mls of Preston broth and incubated at 42 ^{o}C for 48 hrs. under micro aerobic condition. A loopful of the Preston broth culture was streaked on MCCDA and incubated at 42 ^{o}C for 48 hrs as mentioned earlier [14]. Two to three Presumptive colonies of *Campylobacter* isolates were then streaked unto Mueller-Hinton plus *Campylobacter* selective supplement (Oxoid$^{(R)}$ SR 0117 (MHCSS) and incubated as described previously. Biochemical tests for purified positive *Campylobacter* isolates as described in first phase were conducted and stored appropriately before shipment.

On arrival at FARHP, samples were sub cultured on Mueller-Hinton (MH) agar supplemented with *Campylobacter* Selective Supplement (CSS), and incubated under microaerophilic condition for 48hours at 42^{o}C for recovery. Thereafter molecular identification and characterization were conducted on putative isolates.

Polymerase Chain Reaction

DNA extraction was carried out on the isolates by boil preparation method using the protocol described by [15]. Extracted DNA samples were stored at -20^{o}C until further molecular analysis. Multiplex PCR using three target specific primer sets for genotypic identification of *Campylobacter* genus (16*SrRNA*), *C. coli* (*ceuE*), and *C. jejuni* (*mapA*) (Table 1) in a single reaction mixture as described by [16], was performed. The PCR was run in a final reaction volume of 25 µl. PCR conditions used were initial denaturation at 95 ^{o}C for 5mins, 35 cycles of denaturation at 95 ^{o}C for 30mins, annealing at 54 ^{o}C for 60mins, extension at 72 ^{o}C for 2mins and final extension at 72 ^{o}C for 5mins. Control positive strains *C. jejuni* 81-176 and *C. coli* ATCC 33559 were included.

Gel Electrophoresis and DNA sequencing

The PCR products were run in 1% agarose gel stained with 2.5 µl/vol of ethidium bromide. Electrophoresis was performed at 85V for 60 mins and gels were visualized under Ultra violet transilluminator. Some of the isolates that showed amplification with 16 sRNA though not in bands consistent with Campylobacter were sequenced bi-directionally

Phenotypic and genotypic Antimicrobial resistance profile

For detection of four resistant genes (*tet (O)*, *aphA-3-1*, *cmeB*, and *blaOXA-61*) [20,] multiplex-PCR assay was carried out. Also, all the isolates were tested for susceptibility towards five antimicrobial agents, ciprofloxacin, erythromycin, tylosin, spectinomycin, and tetracycline (Sigma-Aldrich Co.) using the broth microdilution methodas described by [21]. Minimum inhibitory concentrations (MIC) and resistance breakpoints were determined.

Statistical analysis

Descriptive statistics were used to describe the prevalence in percentages or proportions with 95% CI. Antibiotic resistance break points were also represented in percentages.

RESULTS

Sequencing Results of Isolates from faeces of chickens in Ogun State

AB-30 was of 79% *C. lari* and 96% *Enterococcus faecalis in* similarity, YW-32 was 80% similar to *C. lari* and 94% *E. coli,* YW-10 was 77% *C. upsaliensis* and 96% *Comamonas kerstersli,* YW-12 was 77% *C. jejuni* and 96% *Comamonas kerstersli* while IJ-12 was 79% *C. lari* and 97% *Pseudomonas aeroginusa*

After bacteriological isolation and molecular characterization, the prevalence of *Campylobacter* from the 50 fecal samples collected showed that 11 were positive as *Campylobacter* (22%), out of these, *Campylobacter coli* was 10(91%) while none was found to be *C jejuni* (0%), and 1 isolate (9%) was another *Campylobacter spp (*Tables 2a and 2b, Fig. 1).

Antibiotic profile and detection of resistance genes of zoonotic Campylobacter in commercial layer chickens

The MIC (phenotypic) results of isolates from farms sampled in Ogun State indicated that all the 10 (100%) zoonotic *Campylobacter coli* isolates were resistant to both tetracycline and spectinomycin, 9/10 (90%) to ciprofloxacin, 8/10 (80%) to tylosin, and 6/10 (60%) to erythromycin (Table 3). Furthermore, all (100%) isolates from poultry farms sampled in Ogun State carried resistant genes of both tetracycline (tet O) and multiclass (cmeB), while none (0%) carried the genes aphA (aminoglycosides) and blaoxa-60(β-lactams).

DISCUSSION

The presumptive prevalence of *Campylobacter* in layer chickens recorded in this study using bacteriological isolation and biochemical characterization was 16.8% which is slightly higher than the prevalence of 12.5 % reported by Adekeye *et al.* [22], in poultry reared under different management systems (deep litter and cage) in Kaduna State. It is however close to 15.4% reported by Olubunmi and Adeniran [23], in Ife, while it is lower than 58% reported by Adegbola *et al* [24], in South-western Nigeria. The method of analysis of the samples in the above-mentioned were bacteriological isolation and biochemical identification methods.

However, using PCR techniques on the presumptive positive isolates in this study, there was no amplification at the expected band size for *Campylobacter species* (875bp). Although *C jejuni* has been reported to be more prevalent in poultry and *C coli* in pigs, our result showed that *C coli* was more in the sampled chickens. This could be as a result of excessive and abnormal use of antibiotics in the study area which prevented the growth of *C jejuni* which is more sensitive to antibiotics as opposed to *C coli* that is more resistant to antibiotics. Also. The farm where positive samples obtained has a pig farm in close proximity to hence the possibility of environmental transmission of *C coli* to the chickens from nearby pigs. After various processes of DNA purification like addition of magnesium chloride at different concentrations, adjusting the annealing temperature, using gradient temperature in thermaocycler as well as the use of dimethyl sulphoxide (DMSO), six of the isolates showed amplification between 920 and 940bp (identified as *Enterococccus coli, Enterococcus faecalis, Comamonas kerstersli* and *Pseudomonas aeroginusa*).This could be that the cultural method of isolation used allowed for contamination during isolation, or that these bacteria grew on the selective medium for *Campylobacter* (mCCDA). Growth media used for laboratory isolation of *Campylobacter* usually contain various selective agents or supplements which enhances *Campylobacter* growth whilst

suppressing other organisms, an example of such is amphotericin B that inhibits growth of fungi and polymyxin B which inhibits the growth contaminating bacterial organisms. However, such agents do not inhibit all microorganisms because other bacteria like *E. coli* and *Enterococcus faecalis* have been reported to grow on most of these selective growth media including the mCCDA (Oxoid®) used in this study. In essence, the inability of the PCR analysis to confirm the organisms isolated as *Campylobacter* could be that some of these contaminating organisms have similar biochemical characteristics with *Campylobacter,* hence their ability to grow on mCCDA. The observation is in agreement with a previous study conducted by Nayak et al. [25], which showed that *cad*F gene amplified in three non-*Campylobacter* strains. To further buttress the possibility of some false positive results occurring, Jamshidi et al. [26] in their study showed that data from cultural and molecular methods revealed huge differences in the detection of *Campylobacter*. Diergaardt *et al*. [27], also in their study in South Africa found that culture isolation on mCCDA were morphologically and biochemically homologous to *Campylobacter jejuni* but organisms found to be *Arcobacter* on further testing using 16sDNA gene sequence as a molecular identification method. This study shows the limitation of conventional cultural method of isolation and biochemical confirmation of *Campylobacter*. Thus, molecular characterization is important in the epidemiology and diagnosis of *Campylobacter* in poultry

High resistance patterns to antibiotics commonly used in poultry farms were recorded in the study, (tetracycline and spectinomycin; 100%, ciprofloxacin; 90%, tylosin; 80% and erythromycin; 60%). All (100%) of the isolates having multiple antibiotic resistance as well as carrying multi-antibiotic resistance genes. This shows that these antibiotics were extensively used in the chickens, most of which may have been without adequate professional supervision.

The result further showed that while all the isolates carried genes resistant to tetracycline and other classes of antibiotics, none carried genes to aminoglycosides and β-lactams groups of antibiotics. This shows that tetracycline is among the frequently used antibiotics in the birds; this may be due to its ease of availability in the study area. Also, ciprofloxacin which may be part of the multiclass resistance group is closely related to enrofloxacin which is regularly used in poultry in Nigeria.

CONCLUSION

The presumptive prevalence of *Campylobacter species* in layer chickens in Ogun State following bacteriological isolation and biochemical characterization is 16.8%.

The prevalence of *Campylobacter spp* in Ogun State following molecular characterization of isolates from improved isolation protocol is 22%.

The conventional isolation protocol of *Campylobacter* was improved on by the addition of maximum recovery diluent and laked horse blood to produce purer culture for molecular characterization.

Table 1. Details of PCR primer sets used for Campylobacter characterization

Bacterial strains	Target gene	Primer sequences	Amplicon size	Reference
Campylobacter Spp	*16sRNA*	*ATCTAATGGCTTAACCATTAAAC* *GGAAGAATTCTGACGGTACCTAAG*	857bp	[17]
Campylobacter Jejuni	*mapA*	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA	589bp	[18]
Campylobacter *coli*	ceuE	*ATTTGAAAATTGCTCCAACTATG* TGATTTTATTATTTGTAGCAGCG	462bp	[19]

Table 2a: Presumptive Prevalence of *Campylobacter species* in isolates from fecal samples of layer chickens in Ogun State

Zone	Number of samples	Number positive (%)	95% CI
Abeokuta	125	27 (21.6)	15.5 – 29.6
Yewa	125	13 (10.4)	6.1 – 17.1
Remo	125	18 (14.4)	9.1 – 21.4
Ijebu	125	26 (20.8)	14.3 – 28.4
Total	500	84 (16.8)	13.8 – 20.3

Table 2b: Prevalence of *Campylobacter species* from samples of layer chickens in Ogun State after molecular typing

Number of sample	Nos (%)positive *Campylobacter spp.*	Nos (%)positive *Campylobacter coli*	Nos (%)positive *Campylobacter jejuni*	Others (%) *Campylobacter*
50	11 (22)	10 (91)	0 (0)	1 (9)

Table 3: Antibiotic resistance profile (phenotypic) of *Campylobacter coli* in faecal samples of layer chickens sampled in Ogun state

Sample Nos	Chlortetracycline	Spectinomycin	Ciprofloxacin	Tylosin	Erythromycin
Total	10/10 (100%)	9/10 (90%)	9/10 (90%)	8/10 (80%)	6/10 (60%)

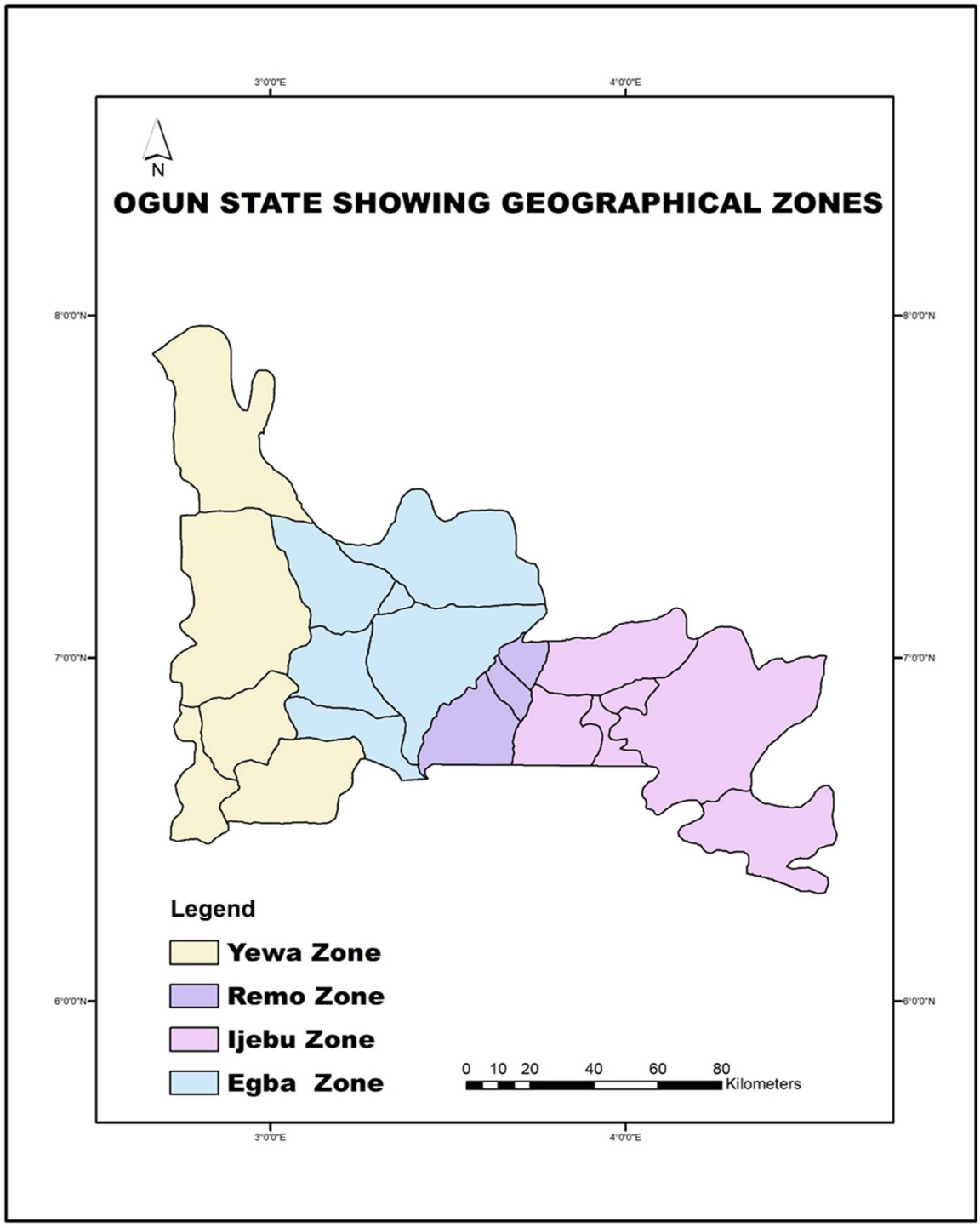

Figure 1: Geographical zones in Ogun State where samples were collected

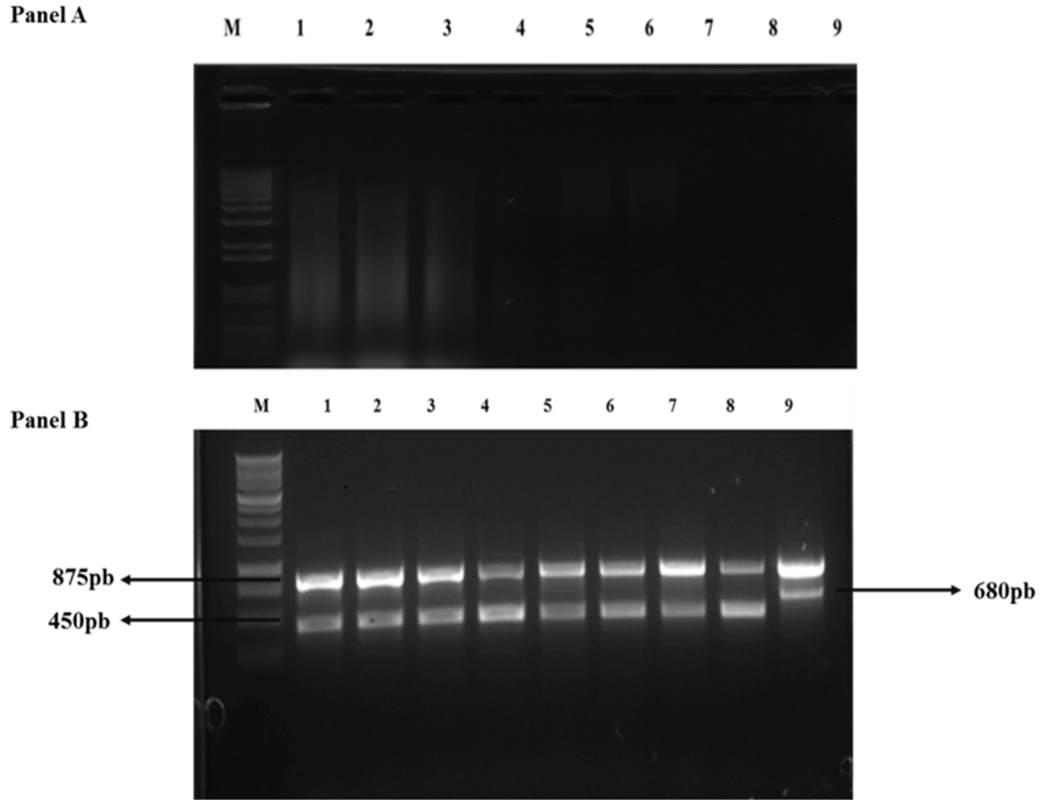

Fig. 2: Panel A shows gel image of PCR analysis of isolates from cloacal swab samples in commercial chickens investigated using isolation method A. No amplification for *Campylobacter spp* was observed. Panel B is gel image of positive bands of *Campylobacter* spp using isolation method B.

M =marker, 1-7: = representing amplicons of DNA from chickens in Ogun State, 8 = control positive strain of C. *coli* ATCC 33559, 9 = control positive strain of *C. jejuni* 81-176. Expected amplicon sizes of 875 bp and 450bp for *C. jejuni* and *C. coli* respectively were observed.

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