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Full Length Research Paper

Isolation and Characterization of *Salmonella* Gallinarum from Outbreaks of Fowl Typhoid in Kaduna State, Nigeria

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This study was carried out to isolate and characterize the causative agent of fowl typhoid in some outbreaks in chickens. Seventy isolates of *Salmonella* Gallinarum were recovered from culturing visceral organs and cloacal swabs obtained from 30 different outbreaks affecting 29 poultry farms in 8 parts of Kaduna State, Nigeria within two years, February, 2011 – January, 2013. Real-Time Polymerase Chain Reaction was carried out using primers targeting *invA* gene which confirmed all isolates as *Salmonella*. Multiplex PCR was carried out to target *glgC* and *speC* genes in order to differentiate between biovars Gallinarum and Pullorum. Results showed that all the isolates possessed both genes, confirming that they were biovar Gallinarum. Ten isolates were selected and characterized by Xbal - Pulsed-Field Gel Electrophoresis (PFGE). The selected isolates represented farms in various parts of the three Local Government Areas (LGAs) where outbreaks occurred. Out of these ten isolates, six isolates were chosen from 55 isolates recovered from 22 affected farms in Sabon Gari LGA. Three isolates were chosen from 9 isolates recovered from 5 affected farms in Kaduna North LGA; while 1 isolate was chosen from 5 isolates recovered from the 2 farms affected in Kaduna South LGA. The PFGE profiles showed >78% degree of relatedness among *Salmonella* isolates. This study provides information that the poultry industry in Kaduna State, Nigeria is still plagued with outbreaks of fowl typhoid caused by closely related *Salmonella* Gallinarum which were isolated.

Key words: Fowl typhoid, *Salmonella* Gallinarum, PCR, PFGE, poultry farms, Kaduna State, Nigeria.

INTRODUCTION

Salmonella Gallinarum causes fowl typhoid in chickens which results in huge economic losses as infected chickens have a high mortality rate, succumbing to septicemia, enteritis, and hemolytic anemia (Shivaprasad, 1997). Fowl typhoid can occur in young as well as older birds (FAO, 2009).

In most countries of Europe and in North America and Australia, fowl typhoid has almost disappeared as a result of improved surveillance and slaughter practices (Christensen et al., 1994; Baumler et al., 2000). However, fowl typhoid has recently been detected on two commercial poultry premises in Northern Ireland after almost 30 years without cases, and was also reported in Great Britain during 2005/2006 following a gap of 20 years (AHVLA, 2012). Fowl typhoid and pullorum disease are serious threats to the poultry industry in Africa, Asia, and tropical Latin America (Baumler et al., 2000). Of all the bacterial diseases that plaque the poultry industry in Nigeria, fowl typhoid and pullorum disease constitute the

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most important ones (Osiyemi, 1976; Onunkwo and Onoviran, 1978; Mbuko et al., 2009). Outbreaks of fowl typhoid are presently being recorded in the study area despite eradication in some parts of the world, therefore the need for the present study as isolation and characterization are first steps towards eradication (Shivaprasad, 2003).

Molecular typing of *Salmonella* has the potential to determine the epidemiology and trace the sources of infection. In this context, Pulsed-field gel electrophoresis (PFGE) is considered a pivotal DNA-based method that complements traditional serotyping (Murase et al., 1995), and is considered the gold standard for molecular sub-typing of *Salmonella* in epidemiological studies (Baggesen et al., 2000). Several molecular techniques have been employed in the characterization of the causative agent of fowl typhoid and reported especially in differentiating between the two poultry serovars, Gallinarum and Pullorum (Kwon et al*.,* 2000; Kisiela et al., 2005; Kang et al., 2011), but such reports are lacking on strains from Nigeria. The present study was therefore, undertaken to identify the specific strains of *Salmonella* Gallinarum causing disease in the chickens in Kaduna State, Nigeria using some of the methods as above.

MATERIALS AND METHODS

Study area and sample collection

The study area was Kaduna State which lies between Latitude 10 ^{o} and 11 ^{o} N; and Longitude 7 ^{o} and 8 ^{o} E in North-Western Nigeria. The present study was conducted from February, 2011 and January, 2013. Three Local Government Areas (LGAs) were selected for the study based on occurrence of fowl typhoid outbreaks affecting the two major cities where poultry farming activities are highest (Poultry Farms Registration Information, Kaduna State). The LGAs were: Kaduna North, Kaduna south, and Sabon Gari Kaduna North had 25 registered farms with an average capacity of 2, 500 birds per farm. In Kaduna South, 25 poultry farms were identified with an average capacity of 1, 500 birds per farm. Sabon Gari had 27 registered poultry farms with an average capacity of about 1, 700 birds per farm (Poultry Farms Registration Information, Kaduna State). The population of birds in these farms ranged from backyard poultry of 200 birds to commercial farms that kept up to 5, 000 birds. All the farms practiced the deep litter management system. During this period a total of 534 samples were collected from both sick and apparently healthy birds at the time of outbreak, and a total of 30 different outbreaks were recorded from 29 farms in these parts of the State. Samples processed from the birds were: intestinal contents, bile (gall bladder content), liver, spleen and ovarian follicles. The samples were all obtained from layers and broilers of all ages. A total of 70 *Salmonella* Gallinarum isolates were recovered from these outbreaks. No vaccination is currently carried out in the study area against *S.* Gallinarum.

Isolation, identification, biochemical characterization and serological tests on the *Salmonella* isolates

Samples such as cloacal swabs from clinically healthy birds; liver, spleen, gall bladder, and caecal contents from sick birds were cultured for isolation and conventional biochemical tests carried out for identification using methods described by Cheesbrough (2006). Fermentation of sugars, such as lactose, sucrose, sorbitol, rhamnose and dulcitol; acid and/or gas production on glucose, fructose, xylose and arabinose incubated at 37 ^{o}C was carried out as previously described (Trabulsi and Edwards, 1962; OIE, 2009). Isolates were confirmed to be *Salmonella* by confirmatory biochemical tests (Cox and Williams, 1976: Ewing, 1986). Serotyping was done by slide agglutination according to Kaufmann-White-Le Minor scheme (Grimont and Weill, 2007) at the Italian and OIE Reference Laboratory for Salmonellosis (OIE and NRL for salmonellosis, Instituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy). Real time PCR was carried out targeting *invA* gene to further confirm isolates as *Salmonella*. All isolates were confirmed as *Salmonella* Gallinarum using multiplex-PCR targeting *glgC* and *speC* genes*,* as previously described (Kang et al., 2011).

Polymerase Chain Reaction (PCR)

All strains serotyped as *S.* Gallinarum were tested by multiplex-PCR targeting *glgC* and *speC* genes*,* as previously described (Kang et al., 2011) with modifications: amplification reaction was carried out in a 50 µl PCR mixture containing 5 µl of 10 x PCR buffer – Buffer Taq Gold (5 µM Tris-HCL, 50 µM KCl, pH 8.8), 4 µl of 2 µM $MgCl_{2,}$ 1 µl of 200 µM dNTPs, 6 µl each of 0.6 µM *glgC* forward and reverse primers; 4 µl each of 0.4 µM *speC* forward and reverse primers respectively; 0.3 µl of 1.5 U Tag Gold DNA Polymerase, 5 µl genomic DNA and 14.7 µl of demineralized water. The PCR was carried out using the following thermal profile: initial denaturation of 5 minutes at 95 ^{o}C followed by 30 cycles, each at 95 ^{o}C for 30 seconds, 60 ^{o}C for 30 seconds, 72 ^{o}C for 30 seconds, with a final extension step at 72 ^{o}C for 5 minutes. The electrophoresis was carried out using 2 % agarose gel, then stained with ethidium bromide solution

Table 1. Distribution of *Salmonella* Gallinarum isolates from poultry farms in Sabon Gari Local Government Area of Kaduna State, Nigeria.

Farm identification	Type of birds kept	Type of sample that tested positive as *S.* Gallinarum	Month and Year of sampling	No. of samples examined	No. (%) of *S.* Gallinarum by serotyping	No. of *S.* Gallinarum by duplex PCR
ABU/1	Layers	Viscera	July, '11	20	1 (5)	1 (5 %)
ABU/2	Layers	Viscera	Feb, '12	20	0 (0)	1 (5 %)
ABU/3	Layers	Viscera	Feb, '12	20	2 (10)	2 (10 %)
ABU/4	Layers	Viscera	Apr, '12	15	1 (6.7)	1 (6.7 %)
ABU/5	Layers	Viscera	July, '12	25	1 (4)	1 (4 %)
ABU/6	Layers	Viscera	July, '12	25	1 (4)	1 (4 %)
ABU/7	Layers	Viscera	Aug, '12	18	1 (5.6)	1 (5.6 %)
ABU/8	Layers	Viscera	Aug, '12	15	1 (6.7)	1 (6.7 %)
ABU/9	layers	Faeces	Aug, '12	15	1 (6.7)	1 (6.7 %)
ABU/10	Broilers	Viscera	Aug, '12	20	3 (15)	4 (20 %)
GRA/1a	Layers	Viscera	Apr, '12	15	3 (20)	3 (20 %)
GRA/1b	Layers	Visc&Faec	Aug, '12	40	25(62.5)	26 (65 %)
Maigana/1	Layers	Viscera	June, '11	20	1 (5)	1 (5 %)
Maigana/2	Layers	Viscera	June, '12	20	3 (15)	3 (15 %)
Sabo/1	Layers	Viscera	Aug, '11	15	1 (6.6)	1 (6.6 %)
Sabo/2	Layers	Viscera	Jan, '12	13	1 (7.7)	1 (7.7 %)
Sabo/3	Layers	Viscera	May, '12	10	1 (10)	1 (10 %)
Sabo/4	Layers	Viscera	Aug, '12	15	1 (6.6)	1 (6.6 %)
Samaru/1	Layers	Viscera	July, '11	10	1 (10)	1 (10 %)
Samaru/2	Layers	Viscera	Aug, '11	15	1 (6.6)	1 (6.6 %)
Samaru/3	Layers	Viscera	Aug, '11	13	1 (7.7)	1 (7.7 %)
Shika/1	Broilers	Viscera	Feb, '11	12	1 (8.3)	1 (8.3 %)
Shika/2	Layers	Viscera	May, '11	15	1 (6.6)	1 (6.6 %)
22 Farms				406	53(13.1)	56(13.8%)

and finally recorded by (DA COMPLETARE CON NOME DEL KODAK).

Pulsed-field gel electrophoresis (PFGE) of *Salmonella* Gallinarum strains

PFGE analysis was performed on a selection of ten (10) *S.* Gallinarum isolates representative of total strains isolated from farms in the three LGAs of Kaduna State where outbreaks occurred. Six (6) isolates were randomly chosen from a total of 55 isolated from 22 affected farms in Sabon Gari LGA. Three (3) isolates were randomly chosen out of 9 recovered from 5 affected farms in Kaduna North LGA; while one (1) isolate was randomly taken out of 5 isolates recovered from the 2 farms affected in Kaduna South LGA. PFGE was carried out after digestion of genomic DNA with the restriction enzyme XbaI according to the Pulse-Net protocol (Ribot et al., 2006). Gel images were analyzed in Bionumerics v5.1 (Applied Maths, Sint-Martens-Latem, Belgium) and compared by cluster analysis using Dice coefficient and Unweighted Pair Group Method with Averages (UPGMA; dendrogram type) with a position tolerance of 1.5 % and optimization of 1.0 %. Fragments that were smaller than 20 kb were not considered for analysis.

RESULTS

Table 1 showed that twenty-two (22) farms were affected in Sabon Gari LGA, 406 samples were processed and isolation rate was 56(13.8 %). In Table 2, five (5) farms were affected in Kaduna North LGA where 76 processed samples yielded 9(11.5 %) isolates of *Salmonella* Gallinarum and in Kaduna South LGA, 50 processed samples yielded 5(10 %) isolation rate (Table 3). From all the tables, a total of 534 samples were examined, 70 Salmonellae were isolated and of these, 66 (12.4 %) were identified as *Salmonella* Gallinarum by serotyping, and 4(0.7 %) were identified as *Salmonella enterica* subsp. *enterica.* Fig. 1 showed a monthly occurrence of

Table 2.Distribution of *Salmonella* Gallinarum isolates from poultry farms in Kaduna North Local Government Area of Kaduna State, Nigeria.

Farm identification	Type of birds kept	Type of sample that tested positive as *S.* Gallinarum	Month and Year of sampling	No. of samples examined	No. (%) of *S.* Gallinarum by serotyping	No. of *S.* Gallinarum by duplex PCR
KDN/1	Layer	Viscera	July, '12	13	1(7.7)	1 (7.7 %)
KDN/2	Layer	Viscera	July, '12	15	0 (0)	1 (6.7 %)
KDN/3	Layer&Broiler	Visc&Faec	Sep, '12	15	2(13.3)	2 (13.3%)
KDN/4	Layer&Broiler	Visc&Faec	Nov, '12	15	2(13.3)	2 (13.3%)
KDN5	Layer	Viscera	Feb, '13	20	3 (15)	3 (15 %)
5Farms				**78**	**8(10.3)**	**9 (11.5%)**

Table 3. Distribution of *Salmonella* Gallinarum isolates from poultry farms in Kaduna South Local Government Area of Kaduna State, Nigeria.

Farm identification	Type of birds kept	Type of sample that tested positive as *S.* Gallinarum	Month and Year of sampling	No. of samples examined	No. (%) of *S.* Gallinarum by serotyping	No. of *S.* Gallinarum by duplex PCR
KDS/1	Layer&Broiler	Viscera	Oct, '12	20	2 (10)	2 (10 %)
KDS/2	Layer	Viscera	Feb, '13	30	3 (10)	3 (10 %)
2Farms				50	5 (10)	5 (10 %)

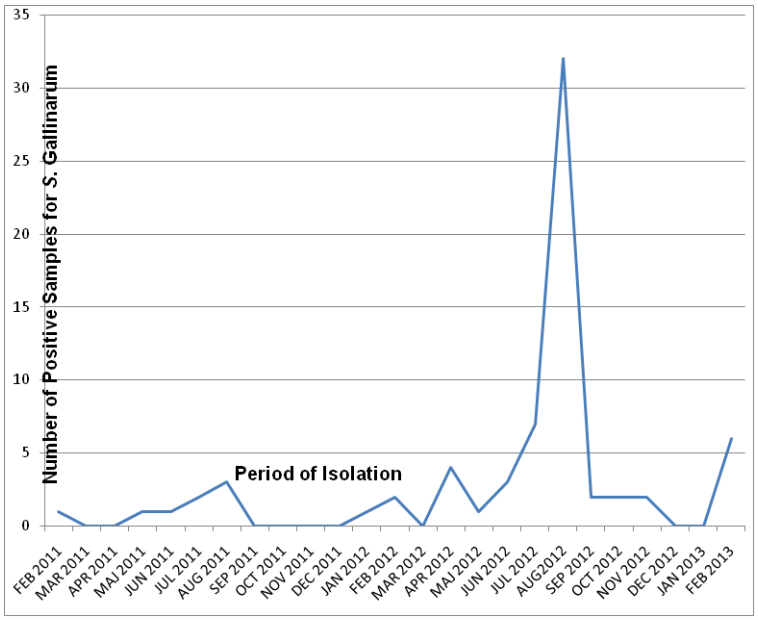

Figure 1. Monthly occurrence of outbreaks of fowl typhoid showing temporal distribution in some poultry farms in Kaduna State, Nigeria.

outbreaks of fowl typhoid where the peaks of occurrence of the outbreaks in the two years of study were observed to appear in the months of July and August. All isolates that tested positive as *S.* Gallinarum were confirmed by

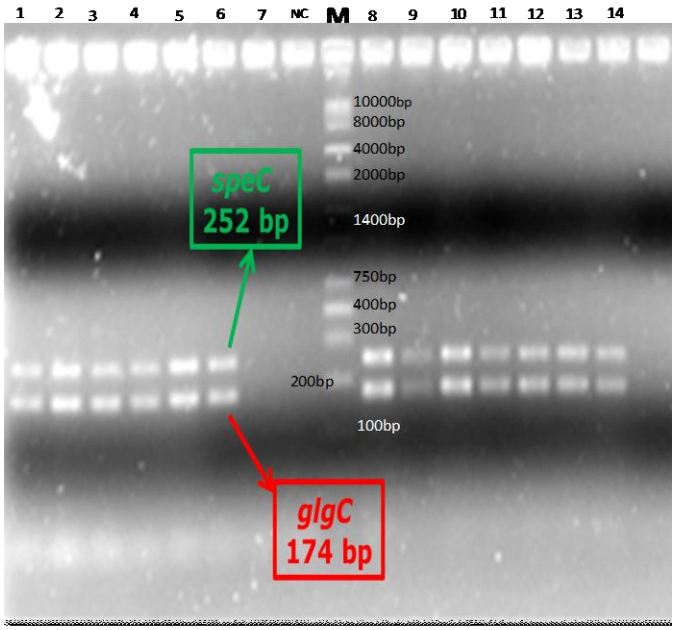

Figure 2: ***Salmonella*** **Gallinarum isolated from some poultry farms in Kaduna, Nigeria; they all amplified** ***speC*** **&** ***glgC*** **genes as shown by the double bands. Nc=Negative control; M=Molecular weight marker**

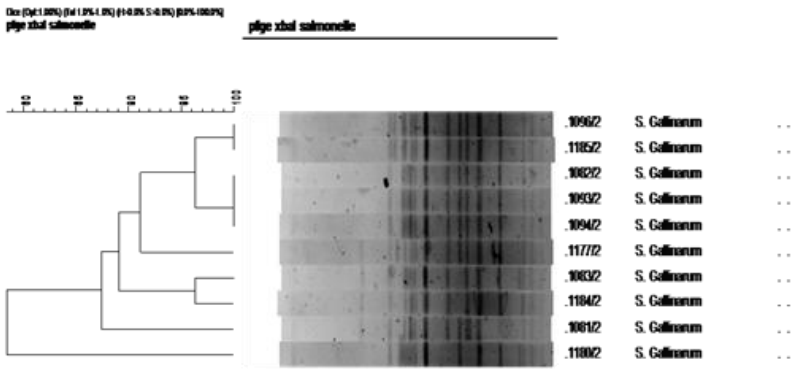

Figure 3.Dendrogram of 10 representative isolates of *Salmonella* Gallinarum strains isolated from poultry in Kaduna State, Nigeria showing strain relatedness of >78 %; constructed based on relatedness within pulsotypes [Analyst Fingerprinting Plus BioRad®)].

multiplex PCR targeting *glgC* and *speC* genes (Fig 2). Ten (10) representative isolates were selected and characterized by XbaI-PFGE typing which revealed six different XbaI profiles within the 10 representative isolates. The overall PFGE profiles of *S.* Gallinarum analyzed by UPGMA showed >78 % level of relatedness. The dendrogram, showing an analysis of the results of the PFGE (Fig 3), revealed that lanes 1 and 2, from the top, were related with 100 % synchrony represented by 1096 for farm ABU3 and 1185 for farm KDS2. The second set of 100 % synchrony was by lanes 3, 4 & 5; which represented 1082 (Shika2), 1093 (KDN1) & 1094 (KDN2) respectively. Lane 6 was represented by 1177 (ABU6), this produced >90 % relatedness with the ones

stated above. Lanes 7 & 8, represented by 1083 (Maigana1) & 1184 (KDS2) showed 100 % synchrony with each other and 89 % with the other groups. Lane 9 was represented by 1081 (Shika1) and was related by >86 %, while lane 10 represented by 1180 (KDN3) had >78 % relatedness with the others.

DISCUSSION

In the course of this study, there were 30 outbreaks of fowl typhoid within the study period which gave an overall prevalence of 13.1 % in 29 affected poultry farms in the study area. The prevalence recorded in this study was considerably low taking into cognizance the fact that the isolation was carried out from outbreaks. This is probably due to the fact that many of the samples were collected from apparently healthy birds on the same farms, and these samples had high chances of yielding negative results. This result is much lower than the prevalence of 37.9 % reported by Okwori et al. (2007) in Jos, Plateau State based on serological detection of antibodies to the organism. This difference is probably because the earlier report by Okwori et al. was a serological study, while this study was based on isolation of the organism. Moreover, the two studies were not carried out exactly in the same area. A retrospective study in one of the regions covered by this study also revealed a higher prevalence of 18.4 % (Mbuko et al., 2009); but this was in relation to other poultry diseases reported.

The rate of isolation of *Salmonella* in this study was observed to be much higher from the visceral organs compared to the faecal samples. It should be noted that out of the visceral samples examined, the highest isolation rate was from the spleen, followed by the liver, the gall bladder, and the ovarian follicles (such details could not be presented in the tables due to cumbersomeness). These findings were most probably due to the stage of the infection as has been reported that during the pathogenesis of *Salmonella*, the bacterial cells in the intestines rapidly invade the lymphoid tissues including the Peyer's patches, the caecal tonsils and probably the enterocytes (Takeuchi, 1967; Turnbull and Richmond, 1978; Propiel and Turnbull, 1985). *Salmonella* organisms would therefore not be isolated easily in the faeces but in the lymphatics and visceral organs instead. Also, survival in macrophages allows *Salmonella* to invade the reticulo-endothelial system (REM), and reside in the liver and spleen (Gray and Fedorka-Cray, 2002), and therefore, this may be responsible for the higher rate of isolation in visceral organs. Faecal samples (cloacal swabs) from apparently healthy birds were directly enriched in selenite broth without pre-enrichment. This factor may explain failure to isolate the *Salmonella* from faeces and may also contribute to the low isolation rate recorded in this study.

In this study, all the isolates of *Salmonella* recovered were confirmed to be *Salmonella* Gallinarum; which could be attributed to host adaptation as *S.* Gallinarum is known to be poultry-adapted (Shivaprasad, 2000). This finding agrees with those of earlier workers (Murugkar et al., 2005; Prakash et al., 2005; Lapuz et al., 2007; Mir et al., 2010) who also isolated only *Salmonella* Gallinarum from different ages of poultry in their studies. Aside host adaptation, isolation of only *S.* Gallinarum in this study could also be attributed to the method of primary isolation that was used. If the 'International Organization for Standardization' ISO 6579 method (EN-ISO 6579:2002/Amd1: 2007) was used, there might have been higher chances of isolating motile serotypes of *Salmonella*, such as *S.* Typhimurium. The bacterium (*Salmonella* Gallinarum) was not breed, sex or age biased as infection was seen to equally affect both layers and broilers and also chicks and adult birds alike being that it was the only *Salmonella* biovar isolated from all the sick birds (just an observation as these parameters were not included in the study design). This is in agreement with earlier reports by Shivaprasad (2000) who stated that *Salmonella* Gallinarum was the causative agent of fowl typhoid in all species of birds and affects both young and older ones.

Even though this study was carried out within a period of two years only, there was an indication that outbreaks occurred mostly during the wet seasons of the year because they were seen to be concentrated between the months of June and August, which are known to record the peak of the rains in the study area (Omogbai, 2010). This assertion agrees with the report on the prevalence and seasonality of fowl typhoid in Zaria-Nigeria reported by Mbuko et al. (2009). This is probably because dampness of the environment encourages the survival of this organism. The results also showed that at the serotyping level, four isolates were identified to the level of *S. enterica* subspecies *enterica,* but these were identified as *S.* biovar Gallinarum at both biochemical and PCR levels. This gives an indication that PCR had a much higher sensitivity for characterization than serotyping. The duplex PCR carried out amplified fragments of the *glgC* and *speC* genes and this has a high power of discriminating between biovars Gallinarum and Pullorum. Biovar Pullorum does not yield amplicon from *speC* gene using the primers that were applied, but biovar Gallinarum yields the products of the two genes using these primers. Kang et al. (2011) used the primer pairs to clearly differentiate between the two biovars with distinct bands.

The PFGE profile of isolates from farms ABU3 and KDS2 (lanes of 1096 & 1185) indicated that the same clonal

type of *Salmonella* Gallinarum was circulating on these two farms. Although these two farms are located in two different parts that are far apart coupled with the fact that the infection occurred at two different seasons of the year; records of the two farms showed that they sourced their chicks from the same hatchery. *S.* Gallinarum infection in these two farms could therefore be attributed to a probable vertical transmission (i.e. trans-ovarian transmission). These findings confirm the role of hatcheries in spreading the *Salmonella* infections as previously established (Kumar et al., 2009; Mir et al., 2010). The second set of isolates showing 100 % synchrony in their PFGE results involved farms Shika2, KDN1 and KDN2 (lanes 1082, 1093 & 1094). It was found that chicks for the two farms (KDN1&2), were sourced from different hatcheries, but Shika2 sourced its chicks from the same hatchery as the first set of two (lanes of 1096 & 1185), which were mentioned earlier above but were sourced in different years. It is possible, therefore, that there was first a vertical transmission to farm Shika2 and by some other means a horizontal transmission to infect the other two farms (KDN1&2). The horizontal transmission could be through feed, water, personnel, or some fomite as two of the farms are located in the same region. Another strain of *S.* Gallinarum from farm KDS2 was also tested and it showed the same PFGE pattern with one from farm Samaru1. Sources of chicks (from farm records), also revealed and incriminated the same hatchery as for Samaru3, KDN2 mentioned above, suggesting vertical transmission. The overall PFGE dendrogram revealed that the strains of *S.* Gallinarum circulating in poultry farms in Kaduna State are likely to be clonally related.

CONCLUSION

The poultry industry in Kaduna State, Nigeria is still plagued with outbreaks of fowl typhoid caused by *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum.

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