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REGULAR ARTICLES

Multidrug-resistant *Salmonellae* isolated in Japanese quails reared in Abeokuta, Nigeria

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Abstract Salmonellosis is a major bacterial disease causing huge economic losses in the poultry industry worldwide. This study was carried out to determine the period prevalence and antimicrobial susceptibility of *Salmonella enterica* in Japanese quails in Abeokuta, Nigeria. Four hundred cloacal swabs of quail birds were collected from 4 locations within Abeokuta. *Salmonella* was isolated from the samples using conventional methods for selective isolation of *Salmonella* and biochemical identification. Isolates were confirmed by polymerase chain reaction assays for the amplification and detection of *Salmonella*-associated virulence genes (*invA* and *stn*) using specific primers. Antimicrobial susceptibility testing was done using the Kirby-Bauer disk diffusion method. In all, *Salmonella* was isolated from 14 (3.5%) cloacal swabs. All 14 isolates possessed *invA* and *stn* genes. The *Salmonella* isolates showed resistance to tetracycline (100%), doxycycline (100%), ampicillin (100%), sulphamethoxazole (92.9%), nalidixic acid (85.8%), ceftazidime (78.6%), neomycin (64.3%), streptomycin (50%) and gentamycin (28.6%) but all the isolates were susceptible to ciprofloxacin. The isolates were resistant to at least three antimicrobials indicating multidrug resistance. The results concluded that Japanese quails harbour multidrug-resistant *Salmonella* which could be transmitted to humans through consumption of contaminated food or by direct and indirect contact with the carrier birds. Antimicrobial resistance could be due to overdependence on antimicrobials. Ciprofloxacin could be considered in the treatment of zoonotic Salmonellosis in humans.

Keywords *Inv a* · Multidrug resistance · Quail · *Salmonella* · *Stn*

Introduction

With an estimated population put approximately 104.3 million, poultry birds represent a major source of high-quality animal protein in Nigeria (Ajala et al. 2007). Poultry production is a very viable business in Nigeria with great potentials for growth and income generation. The livestock industry in Nigeria provides about 36.5% of the total protein intake of Nigerians and poultry contributes substantially to this (UNDP 2006).

Over the years, there has been a significant gap between the production and demand of animal protein to feed the ever growing population. To halt this negative trend, efforts are being directed towards improving the livestock industry with the introduction of micro-livestock possessing prolific tendency, short gestation period, short generation interval and rapid growth (Owen and Amakiri 2010). Among the micro-livestock animals is the Japanese quail (*Coturnix coturnix japonica*). Japanese quails are hardy birds that thrive in small cages and are cheap to produce. They have less feed requirement of about 20–25 g feed per day compared to chicken that requires 120–130 g per day (Ani et al. 2009). The Japanese quail attains market weight of 140–180 g between 5 and 8 weeks of age and have a high rate of egg production between 180 and 250 per year (Shwartz and Allen 1981; Garwood and Diehl 1987).

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Salmonellosis is considered a major bacterial disease problem in the poultry industry worldwide. *Salmonella* is one of the important inhabitants of the gastrointestinal tract that causes foodborne infection worldwide, resulting in millions of human infections and annual deaths (Mead et al. 1999). *Salmonella enterica* are carried by a wide range of food and wild animals, rodents, pets, birds, reptiles and insects, usually showing no clinical signs (Adams and Moss 1999). Most of the *Salmonella* infections in humans' results from the ingestion of contaminated poultry and poultry products (Carli et al. 2001).

In humans and veterinary medical practices, the use of antibiotics in the treatment of life-threatening bacterial diseases has reduced mortality rates and improved productivity especially in food animals over the past decades. But recently, antibiotic resistance has become a serious public health and food safety problem with the emergence of multidrug-resistant bacterial species in man and animals. The worldwide increase in the use of antibiotics, as an integral part of the poultry and other livestock production especially for the treatment and prevention of bacterial diseases and as growth promoter has contributed to the emergence of multidrug-resistant bacteria in recent years (Oluwasile et al. 2014). A recent report by the Centre for Diseases Control and Prevention (CDC) revealed that antimicrobial susceptibility data on *Salmonellae* are scarce in West Africa including Nigeria and as such treatment of salmonellosis with appropriate antibiotics is not usually done. This could encourage the development of multidrug resistance among *Salmonella* serotypes (CDC 2015).

This study investigated the prevalence and antimicrobial susceptibility of *Salmonella* in apparently healthy Japanese quails reared in Abeokuta.

Materials and methods

Sample collection

Four hundred cloacal swabs were randomly sampled from selected quail farms in Abeokuta, Ogun State. Samples were collected aseptically using sterile swab sticks to scoop faeces from the cloaca of the birds and placed in sterile universal bottles. One hundred samples from two farms were collected from each of the four different locations (Camp, Olomore, Elega and Obantoko). Samples were transported in container with ice packs to the laboratory for microbiological examination.

Isolation and identification *Salmonellae*

Conventional methods for the detection of *Salmonella* were carried out according to the procedure described by Davies and Wray (1994). Briefly, each cloacal swab was pre-enriched in 9 ml of buffered peptone water and incubated at 37 °C for 24 h. The pre-enrichment broth culture was mixed and 1 ml was transferred with a sterile pipette into a tube containing sterile 9 ml of Mueller-Kaufmann Tetrathionate novobiocin (MKTTn) broth. Moreover, 0.1 ml of pre-enrichment broth culture was transferred in drops onto 9 ml of sterile Modified semi solid Rappaport Vassiliadis agar (MSRV). Inoculated MKTTn broth was incubated at 37 °C for 24 h and the MSRV was incubated at 41.5 °C for 24 h. After incubation, a loop full of MKTTn and MSRV cultures were streaked separately onto the surface of Xylose lysine desoxycholate (XLD) agar and MacConkey agar (MAC). The plates were incubated at 37 °C for 24 h. After incubation, the plates were checked for growth of typical *Salmonella* colonies. Typical colonies of *Salmonella* grow on XLD agar with a black centre and a lightly transparent zone of reddish colour due to the colour change of indicator, while typical colonies of *Salmonella* grows on MAC agar as pale translucent colonies and slightly convex. Identification of suspected *Salmonella* colonies was based on morphological, cultural and biochemical tests as previously described (Barrow and Feltham 1995; Shivaprasad 2000).

Molecular confirmation of *Salmonellae*

Genomic DNA was extracted from 1-ml overnight tryptic soy broth culture of each isolate using the QIAamp ® DNA Mini extraction kit (QIAGEN, USA). The extracted DNA was used for the detection of *stn* (*Salmonella* enterotoxin), and *inv. A* (invasive) gene by PCR analysis (Rahman 1999; Rahman et al. 2000).

Primers used for *stn* gene were Stn P1 5-TTG TGT CGC TAT CAC TGG CAA CC-3 (upper primer) and *stn* M13 5-ATT CGT AAC CCG CTC TCG TCC-3 (lower primer)10 (GENSET, Singapore), which flank a 617 bp segment in the *stn* gene sequence. The PCR mixture (25 μl) included 12.5 μl master mix (QIAGEN, USA) containing 2.5 U Taq DNA polymerase, 20 μM each of dATP, dCTP, dTTP and dGTP, $MgCl_2$ and PCR buffer, 5 μl (1 μM) each of upper and lower primers and 2.5 μl of template DNA (from the isolate). PCR reaction was performed in a thermocycler (Biorad, icycler) in 25 cycles of denaturation (94 °C for 1 min), primer annealing (59 °C for 1 min) and primer extension (72 °C for 1 min) followed by final elongation at 72 °C for 10 min.

For *invA* gene, the primers used were S139-5 GTG AAA TTA TCG CCA CGT TCG GGC AA-3 (upper primer) and S141-5 TCA TCG CAC CGT CAA AGG AAC C-3 (lower primer). These primers flank a 284 bp segment in the *invA* gene sequence. Reactions with these primers were carried out in a 25 μl amplification mixture consisting of 12.5 μl of PCR buffer, dNTPs (10 mM) and $MgCl_2$ (50 mM), 5 μl of each primer, 0.5 μl of Taq DNA polymerase (fermentase) and 2 μl of extracted DNA (Soumet et al. 1999). The amplification condition was as follows: an initial incubation at 95 °C for

5 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 56 °C for 30 s, elongation at 72 °C for 30 s and final extension period for 10 min at 72 °C.

Then, 15 μl aliquot of each PCR product was electrophoretically separated on agarose gel (1%, containing 0.5 μl/ml ethidium bromide, Pharmacia, Sweden) at 100 V for 1.5 h and molecular size marker of 100 bp was added to the gel. It was visualised under UV light (300 nm) from the transilluminator and photographed using Gel doc 2000 documentation system (Pharmacia, Sweden).

Antibiotic susceptibility testing

Antibiotic sensitivity test was conducted using antibiotic discs (Oxoid, UK) according to Kirby-Bauer antibiotic disc diffusion techniques. Briefly, Mueller-Hinton agar was prepared in Petri-dishes. Pure colonies of the isolated organisms were resuscitated in normal saline and the turbidity matched against 0.5 McFarland turbidity standard. The bacteria were plated on the Mueller-Hinton agar and antibiotic disc was placed centrally using the antibiotic disc dispenser (Oxoid, UK). The Petri-dish and its content were incubated for 18 h at 35 °C. The organisms were observed for antibiotic sensitivity by measuring the zone of inhibition on the plate (Baker and Breach 1980).

Statistical analysis

Descriptive statistics were used to describe the prevalence analyses and antibiogram was presented in percentages. Programs Excel version 2003 (Microsoft® Office Excel 2003) and Statistical Package for Social Sciences (SPSS. 16) were used for data collection, management and analysis.

Result

In this study, *Salmonella* was isolated from 14 (3.5%) out of the total 400 cloacal samples examined as shown in (Table 1). Camp has the highest prevalence of 6%, followed by Olomore 5%, Obantoko 3% while *Salmonella* was not detected in any of the samples from Elega.

Table 1 Prevalence of *Salmonella* in cloacal swabs of Japanese quail from selected quail farms in Abeokuta

Locations	No. of samples	No. of positive (% prevalence)
Camp	100	6 (6)
Obantoko	100	3 (3)
Elega	100	0 (0)
Olomore	100	5 (5)
Total	400	14 (3.5)

Antibiotic susceptibility of *Salmonella* isolates from cloacal swabs of Japanese quails in Abeokuta

Antibiotic resistance rates of the 14 *Salmonella* isolates are shown in (Table 2). The isolates were resistant to ampicillin (100%), doxycycline (100%), tetracycline (100%), sulphamethoxazole (92.9%), nalidixic acid (78.6%), ceftazidime (78.6%), neomycin (71.4%), streptomycin (57.2%) and gentamycin (21.4%). All isolates were susceptible to ciprofloxacin. The isolates showed divergent resistance pattern with most isolates showing resistance to at least four out of the ten antibiotics tested (Table 3).

The 14 salmonella isolates were positive for *invA* and *stn* genes by polymerase chain reaction method.

Discussion

In this study, 14 (3.5%) of 400 cloacal swab samples examined were positive for *Salmonella*. The 3.5% prevalence of *Salmonella* observed in the cloacal swabs of Japanese quail in this study was higher than 2.98% in Japanese quail in Iran (Jahantigh et al. 2012) and 0% in Japanese quail in Brazil (Teixeira et al. 2013) but lower than 9% in quail birds in Egypt (Maysa et al. 2013). This may be due to the difference in the geographical locations, lack of farms biosecurity measures, bad management practices as well as the ability of *Salmonella* to survive on the wide range of animate and inanimate carriers. Salmonellosis remains a major foodborne disease in human. The significance of *Salmonella* spp. as the aetiology of human and animal diseases has increased over the years. Modern practices in the poultry industry are now more favourable for the maintenance and dissemination of *Salmonella* serotypes (Harsha et al. 2011).

In the present study, the resistance pattern of the 14 *Salmonella* isolates to the 10 antibiotics tested showed that

Table 2 Antibiotic resistance patterns of *Salmonella* isolates from cloacal swabs of Japanese quails in Abeokuta

Antibiotics	Code	Disc conc μg/disc	No. of isolates resistant (%)
Gentamycin	GEN	10	3 (21.4)
Doxycycline	DOX	5	14 (100)
Ciprofloxacin	CIP	5	0 (0.0)
Tetracycline	TET	30	14 (100)
Neomycin	NEO	10	10 (71.4)
Ceftazidime	CAZ	25	11 (78.6)
Ampicillin	AMP	25	14 (100)
Streptomycin	STR	10	8 (57.2)
Nalidixic acid	NAL	10	11 (78.6)
Sulphamethoxazole	SUL	300	13 (92.9)

Table 3 Antimicrobial resistance patterns of *Salmonella* isolates from cloacal swabs of Japanese quails in Abeokuta, Ogun State

Number of antibiotics	Resistance patterns	Number of multi resistant isolates
4	AMP DOX SUL TET	1
5	AMP DOX NAL SUL TET	2
5	AMP CAZ DOX SUL TET	1
6	CAZ DOX NAL NEO SUL TET	1
7	AMP CAZ DOX NAL NEO STR TET	1
7	CAZ DOX GEN NAL NEO SUL TET	1
7	AMP CAZ DOX NEO STR SUL TET	1
8	AMP CAZ DOX NAL NEO STR SUL TET	3
9	AMP CAZ DOX GEN NAL NEO STR SUL TET	3
Total		14

the bacteria have acquired resistance to most of the antibiotics. This may be due to indiscriminate use of antibiotics in poultry production that has provided selective pressure for the emergence of drug-resistant bacteria associated with poultry (Gunner et al. 2004). The *Salmonella* isolates in this study were all resistant to tetracycline. This is similar to the reports of Fashae et al. (2010) who observed 93% *Salmonella* resistance to tetracycline in Ibadan, Oyo State but higher than the 55.26% resistance reported by Oyekunle et al. (2003) in *Salmonella* isolates from Yewa division of Ogun State. Thus, it appears there is an increased trend in salmonellae resistance to tetracycline over time within the same geographical location. Jones and Ricke (2003) reported that in Nigeria, tetracycline is often administered in poultry as prophylaxis, growth promoter or for the treatment of infections all through life. There is evidence to indicate that tetracycline has bioaccumulation action compared to other antibiotics, which may be critical in maintaining the tetracycline resistance at a high level (Frost 1991). Antibiotics (including the tetracyclines) are administered usually during the first few days of life and occasionally when mortality occurs. Hence, poultry birds are exposed to tetracyclines especially chlortetracycline and oxytetracycline from day-old up to slaughter stage.

In this study, the salmonellae were 100% susceptible to ciprofloxacin, which is a member of the fluoroquinolones. This finding disagrees with the report of Harsha et al. (2011) who observed 16.6% resistance of *Salmonella* isolates to ciprofloxacin from laying birds but agrees with the reports of Singh and Gupta (1999) who observed considerable variation in the resistance pattern of different isolates of *Salmonella* with 100% sensitivity to ciprofloxacin. This high susceptibility may imply that the salmonellae have not acquired the resistance to fluoroquinolones. Moreover, this drug is rarely used in the treatment of infections in quail birds, unlike in commercial chickens where the drug has been used frequently in the treatment of salmonellosis and other bacterial infections (Fashae et al. 2010). This also means that ciprofloxacin may be a good drug of choice for the treatment of infections caused by salmonellae provided it is adminisered judiciously only when absolutely needed.

Carramiñana et al. (2004) observed 96.2% sulfadiazine resistance in *Salmonella* originating from poultry slaughterhouse in Spain similar to the 92.9% resistance to sulphamethoxazole observed in this study. However, Fashae et al. (2010) observed a relatively lower *Salmonellae* resistance of 87% to sulphamethoxazole. Resistance to sulphamethoxazole may have resulted from selective pressure resulting from the use of sulfaquinoxaline (a closely related sulphonamide) in combination with amprolium (anticoccidial drug) as a broad-spectrum anticoccidial drug. This drug combination is marketed commercially (e.g. embarcine forte®) and is probably the commonest drug in the study area for the prevention and control of coccidiosis in poultry birds including quails.

The 85.8% resistance to nalidixic acid observed in this study is higher than 20% resistance in salmonella from quails in Egypt (Ashraf et al. 2015) and 11.11% in salmonellae from quail eggs in India (Harsha et al. 2011). This drug is a first-generation quinolones that is very effective in the treatment of infections in both man and animals and has been used in monitoring the degree of resistance to fluoroquinolones (Ackers et al. 2000). The high resistance observed in this study may be due to a step-wise progression from low-level to high-level resistance which occurs in bacteria through sequential mutations in chromosomes (Schneiders et al. 2003). Salmonellae and other *Enterobacteriaceae* have evolved increasing resistance to fluoroquinolones, as a result of mutations in the target enzymes (topoisomerases) and an increase in the expression of membrane proteins that pump the drugs out of the cells (Wang et al. 2001). There was 21.4% resistance to gentamycin, an aminoglycoside commonly used in the treatment of bacterial infections. This is higher than the 0% resistance to gentamycin in salmonellae from quail eggs reported in India (Harsha et al. 2011) and 16% in salmonellae from chickens in Nigeria (Ike et al. 2014) but lower than 100% in salmonellae from humans in Kogi State, Nigeria (Sule et al. 2012).

The high resistance of 78.6% to ceftazidime (a third-generation cephalosporins) observed in this study was higher than 0% resistance reported in quail reared in Egypt (Ashraf et al. 2015). Cephalosporins are members of an important class of antibacterial agents (beta lactams) used in the treatment of serious salmonellosis in both humans and animals (Cavaco et al. 2008). The use of cephalosporins in food-producing animals could be a selective factor for the appearance of extended spectrum β-lactamases (ESBL)-producing and multiple-antimicrobial-resistant bacteria in animals

(Cavaco et al. 2008). A close attention should be given to the members of *Enterobacteriaceae* as they pose threat to human health (Okesola and Makanjuola 2009). *Salmonella* are among the notable bacteria known to carry plasmids, which encode for drug resistance. This implies that widespread use of antimicrobials in animals or humans may cause an increase in the frequency of occurrence of bacteria resistant to other antimicrobials as the resistance (R) plasmid may encode resistance to additional antimicrobial agents (Molla et al. 2003). There are several reports on the trend of infection through faeces, suggesting that *Salmonella* species and *Escherichia coli* are the main carriers of antimicrobial resistance genes in faecal flora of animals and human (Carattoli 2003; Helms et al. 2005; Osman et al. 2006).

Resistance genes are often located on extra-chromosomal genetic elements or in segments inserted within the chromosome that originates from other genomes (Carattoli 2003; Yah et al. 2007). The acquisition of a new gene may occur by genetic transformation or through mobilization by conjugative transfer. The latter may occur at high frequency and efficiency, and several resistance genes can be acquired simultaneously (Carattoli 2003). According to Carattoli (2003) and Yah et al. (2007), the antibiotic resistance in those isolates which seem not to possess plasmids was associated with chromosome and/or transposons instead of being plasmid-mediated. The transferred plasmids DNA varied among the *Salmonella* species. The exchange of plasmid(s) between bacterial cells and the integration of resistance genes into specialized genetic elements play a major role in acquisition and dissemination of antibiotic resistance genes among the *Salmonella* species (Winokur et al. 2000; Carattoli 2003; Helms et al. 2004; Osman et al. 2006; Yah et al. 2007).

In this study, the 14 *Salmonella* isolates were all positive for the *invA* gene tested which correlates with the phenotypic findings (appearance on selective medium and biochemical tests). This gene which is specific for *Salmonella* chromosomally located aids attachment of the pathogen to the epithelial cells allowing for the penetration of the host cells leading to spread and destruction of those cells (Rahman et al. 2000).

The 14 Salmonella isolates were also positive for *stn* (*Salmonella* enterotoxin) gene. *Salmonella*-induced diarrhoea is a complex phenomenon involving several pathogenic mechanisms including production of enterotoxin (Rahman et al. 2000). This enterotoxin production is mediated by the *stn* gene. This factor is important in the pathogenicity of *salmonellae*.

Most of poultry farmers in the study locations have been administering antibiotics to their quails indiscriminately without veterinary supervision. The availability of chickens in the same farms with quails could also promote transmission of *Salmonella* from chickens to quail or vice versa. It was observed that the same farm attendants are involved in caring (feeding, watering and other cares) for both the chickens and quails on these farms. Thus, the attendants may acts as a vehicle for the transmission of *Salmonella* between the two species of birds.

Conclusion

The prevalence of multidrug-resistant Salmonella in Japanese quails reared in Abeokuta poses public health risk. This is of serious concerns in that the antibiotic options available for treatment is being limited and narrowed. Moreover, it could be speculated that bacteria will eventually acquire resistance to the antibiotics to which they are currently susceptible if the excessively and indiscriminately use are not restricted by strict regulations and enforcement. The prophylactic use of many antimicrobials in poultry feed can also lead to acquired antibiotic resistance. Strict hygiene, good management practices and judicious use of antibiotics are possible ways of preventing diseases and emergence of multidrug-resistant bacteria.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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