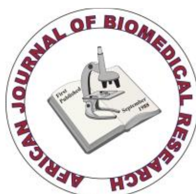

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*Afr. J. Biomed. Res. Vol.20 (January, 2017); 53-58*

*Full Length Research Paper*

# Characterization of a Multidrug Resistant *Salmonella Enterica* Give Isolated from a Lizard Captured in a Poultry House in Nigeria

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**ABSTRACT**

*Salmonella enterica* Give is one of the serotypes that have been incriminated in Salmonella infections; sometimes associated with hospitalization and mortalities in humans and animals in some parts of the world. In this work, we characterized one *Salmonella* Give isolated from cloaca swab of an *Agama agama* lizard captured from one of the commercial poultry pens in Nigeria; where lizards often have access to poultry feeds and water. The isolate was characterized based on conventional morphological and biochemical bacteriological procedures, serotyping, and PCR based Sip C gene screening. Based on the antibiotic resistant patterns of the isolate, it was further screened for the presence of point mutation at the gyrA subunit of the quinolone resistant determining region, for the presence of Salmonella Genomic Island 1(SGI1) integron related genes, and for bla- NDM-1 gene using PCR assay. The isolate exhibited resistance to: ceftazidime, ceftriaxone, amikacin, cefepime, levofloxacin, sulfamethoxazole, chloramphenicol, kanamycin, ampicillin and streptomycin at the respective breakpoint concentration. It had a high MIC of >128µg/mL for Levofloxacin with one point mutation of H150Y substitution. The serotype did not however carry any of the SGI 1 related integron genes tested, neither does it bear the bla- NDM-1 gene despite its phenotypic resistance to ceftazidime, ceftriaxone, cefepime and amikacin at the breakpoint concentrations of 32µg/mL. This finding shows that *Agama agama* lizards can constitute a public health threat as agents of spreading the drug resistant serotype to poultry and humans.

Keywords; Salmonella enterica Give; lizard; public health; poultry.

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*Received: January, 2016; Accepted: June, 2016*

***Abstracted by:***
*Bioline International, African Journals online (AJOL), Index Copernicus, African Index Medicus (WHO), Excerpta medica (EMBASE), CAB Abstracts, SCOPUS, Global Health Abstracts, Asian Science Index, Index Veterinarius*

## INTRODUCTION

*Salmonella* species are members of the family *Enterobacteriaceae*, and they are facultatively anaerobic, non-spore forming, Gram-negative rods (Holt et al., 1994). They catabolized a number of sugars such as D-glucose with the production of acid and usually gas, they are oxidase negative, catalase positive, indole, Voges-Proskauer negative, methyl red and Simmons citrate positive, some produce $H_2S$; and they do not hydrolyze urea (Holt et al.,1994; Lightfoot, 2004; Percival *et al*.,2004).The infection caused by *Salmonella species,* in human and animal, is called Salmonellosis; where infected human may develop diarrhea, fever and abdominal cramps within 12-72 hours following infection (CDC 2003). *Salmonella enterica* comprising a large number of serotypes is of great public health importance because they are often associated with food-borne disease (Weil *et al.,* 2004). Salmonella is considered one of the most important animal-related zoonosis found worldwide in poultry, pigs and cattle (Anderson *et al*., 1999; Lo Fo Wong. 2002). In a report on estimation of illnesses, hospitalization and death through pathogens in the USA between 2000 and 2008, the annual illnesses due to non-typhoidal *Salmonella species* was 1000,000; 19000 hospitalization and 380 death; while 1800 illnesses, 200 hospitalization and no death were reported for *Salmonella enterica* serotype Typhi (Scallan et al., 2011).

The danger and possibilities of bacteria disease transmission and particularly, Salmonellosis through reptile is well appreciated, and this has led to precautionary step to preventing this in some parts of the world (Anonymous,

2015). For example, U.S. Food and Drug Administration enforced ban on the sales or distribution of small turtles with shells that measure less than 4 inches in length, because of the possibilities of children putting them in their mouths as toy since 1975 (Anonymous, 2015). Right from 1970s,captive reptiles like tortoise and turtles were documented to be more involved with the transmission of *Salmonella enterica* than lizards, but the trend seems to have changed with more prevalence in lizards than other reptiles since year 2000 following (Chiodini et al., 1981; Kikillus et al., 2011; Willis et al., 2002; Anonymous, 2002; Pasman et al.,2005; Kaibu et al., 2006; Weiss Hernández et al., 2012).There was resurgence of reptiles-associated salmonellosis since, 1990s to 2000 for example, in USA(Woodward et al., 1997; Warwick et al., 2001; Marmin et al., 2004).Since 2006, CDC received reports of 11 multistate outbreaks, including 6 ongoing outbreaks, and more than 535 cases of laboratory-confirmed *Salmonella* infections linked to contact with small turtles and their habitats. The illnesses resulted in about 85 hospitalizations and one death (Anonymous, 2015).

*Salmonella* Give is one of *Salmonella* enterica serotypes that has been reported in human and animal related salmonellosis in different parts of the world. For instance, *Salmonella* Give associated salmonellosis was reported in two neighboring dairy farms from Eastern Township of Quebec. The first case in a farm started with a cow presented with profuse diarrhea and hypocalcemia, the case was later confirmed to be widespread in the herd and *Salmonella* Give was isolated from bulk milk samples as well as from intestines of 2/5 of euthanized cat often seen in the feeding alley in barns of the farm (Roy et al., 2001). A similar case was likewise, observed in a neighboring dairy farm, where *Salmonella* Give 3/10 was also isolated, but it was not wide spread within the second herd compared to 22/61(41%) as observed in the first case, and the organism was not isolated from the intestine of the euthanized cat (Roy et al., 2001). Prior to the report by Roy et al., 2001, only one isolation of *S*. Give had been reported in Quebec between 1990 and1995. In 1996, an outbreak involving 7dairy herds in different regions of the province was associated with the same serovar (Haggin et al., 1997). Molecular typing analysis using pulsed-field gel electrophoresis (PFGE) showed that all isolates had the same PFGE type, and phage typing also indicated that they belonged to the same phage type(Stl,3/Si2/Sn3) (Haggin et al., 1997). *Salmonella* Give was also isolated from Salmonella infection involving 3 infants suspected to contact the infection through common brand of milk formula they drank from West France (Jourdan et al., 2008). The report from the database of the French National reference Centre (NRC) for Salmonella likewise indicated the 5/6 of similar *Salmonella* Give related cases in infants around the same time was traceable to the same milk formula consumption (Jourdan et al., 2008).

In Nigeria, a *Salmonella* Give was isolated from the small intestine of a parrot from Kano zoological garden about two decades ago (Okon and Onazi,1980). More recently, two levofloxacin resistant *Salmonella* Give that possessed gyrA mutation encoding histidine to Tyrosine conversion at amino acid 150(150His→Tyr) had been earlier isolated and characterized, one from septic poultry and one from asymptomatic pig (Ogunleye et al., 2011). The pig isolate possessed an additional (83Tyr→Ser) substitution (Ogunleye et al., 2011). To the best of our knowledge, this work reports the first isolation and characterization of *Salmonella* Give from cloaca swab of lizards captured in a poultry house in Ibadan, Nigeria. The isolate was characterized based on morphological, biochemical, serotyping, PCR screening for sip C gene; amplification of gyrA subunit of the quinolone resistant determining region, Salmonella genomic island 1integron screening and the presence of NDM-1 gene based on the antibiotic resistant pattern of the isolate.

## MATERIALS AND METHODS

**Salmonella isolate**: The *Salmonella* characterized was isolated from a cloaca swab of one *Agama agama* lizard captured in a commercial poultry farm in Ibadan, Oyo State, Nigeria. The isolate was tentatively identified as *Salmonella enterica* based on morphological, biochemical and serological typing with Polyvalent Salmonella antisera (Difco m Salmonella O Antiserum Ploy A-I and V1 (Edwards and Ewings, 1972; Barrow and Feltham, 1993).

**Serotyping of the isolate:** The isolate was sub cultured into TSA agar and submitted to National Veterinary Service Laboratories in Ames, Iowa State, USA for serotyping. The serotyping was performed based on Kauffmann White Scheme.

**Identification of the isolate with Sip C PCR assay:** The identity of the isolate was confirmed as Salmonella in a PCR assay with Sip C F- 5´- ACAGCAAAATGCGGATGCTT-3´ and Sip C R- 5´- GCGCGCTCAGTGTAGACTC-3´ as earlier described by Carlson et al., 1999 with slight modification. Chromosomal DNA was produced from the isolate by heating the LB broth cultures at 99$^0$C for 15minutes. A 100µl of the boiled isolate was mixed with equal volume of PCR grade water, 1 µl of the mixture was used as DNA template in a 50 µl reaction. The DNA was amplified using QS PCR reagents containing 1µM of Sip C F- 5´- ACAGCAAAATGCGGATGCTT-3´ and Sip C R- 5´- GCGCGCTCAGTGTAGACTC-3´ including 10 µl QS buffer, 1 µl dNTPs, 0.25 µl Sip C F , 0.25 µl Sip C R , 0.5 µl QS enzyme, 10 µl QS enhancer and 27 µl PCR water, using the PCR protocol: with initial denaturation at 98$^0$C for 30 seconds, then 35cycles of 98$^0$C for 10seconds, 60$^0$C for 30seconds, 72$^0$C for 15seconds and 72$^0$C for 1minute, 55 seconds. One *Salmonella* Kentucky earlier isolated from septic poultry from Nigeria by Ogunleye and Carlson, 2012 was used as positive control. The amplified products were resolved with precast E- gel in an Electrophoresis unit (Life Technologies).

**Determination of Resistance to Kanamycin, ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, levofloxacin, ceftazidime, ceftriaxone, cefepime and amikacin:** The isolate was grown aerobically in breakpoint concentrations of 32µg/mL each for kanamycin, ceftazidime, ceftriaxone, amikacin, ampicillin, and cefepime; at 64 µg/mL for streptomycin, 16 µg/mL for chloramphenicol, sulfamethoxazole at 1,024µg/mL and 8µg/mL for

levofloxacin (all from SIGMA- ALDRICH) according to standard method( CLSI, 2009). Resistance was ascribed if flocculent growth was observed after 16h of aerobic growth at 37$^0$C.

**Salmonella genomic island (SGI 1) Integron screening:** Based on the isolate's resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline at their respective breakpoint concentrations, the isolate was screened for the presence of SGI1 genes by using CmlAtetR R- 5′-GCTGCGTTCATCTACAACAGAT3′ and CmlAtetR F 5′-CGCTCCTTCGATCCCGT 3′ as earlier described by Carlson et al., 1999. One integron positive Salmonella Kentucky earlier isolated from septic poultry in Nigeria by Ogunleye and Carlson, 2012 was used as positive control.

**NDM- 1 gene screening:** Based on the *Salmonella* isolate's resistance to ceftazidime, ceftriaxone, cefepime and amikacin at the respective breakpoint concentrations, the isolate was screened for the presence of bla$_{NDM-1}$ by PCR as earlier described (Chen et al., 2011), with some modifications. The isolate was screened with primers NDM- 1 F-(5′-ATGGAATTGCCCAATATTAT-3′) and NDM- 1 R-(5′-TCAGCGCAGCTTGTCGGCCA-3′). 1 µl of the boiled crude chromosomal DNA was used as template in a 50 µl reaction. The DNA was amplified using QS PCR reagents containing 1µM of NDM- 1 F(5′-ATGGAATTGCCCAATATTAT-3′)and NDM- 1 R-(5′-TCAGCGCAGCTTGTCGGCCA-3′), including 10 µl QS buffer, 1 µl dNTPs, 0.25 µl NDM 1- F, 0.25 µl NDM-1 R, 0.5 µl QS enzyme, 10 µl QS enhancer and 27 µl PCR water, using the PCR protocol: 98$^0$C for 30 seconds, 35cycles of 98$^0$C for 10seconds, 55$^0$C for 30seconds, 72$^0$C for 1minute 15seconds and 72$^0$C for 7minute. The synthesized DNA manufactured by Genscript$^R$ with the Oligosequence shown in Figure 1 and one *Pseudomonas aeruginosa* isolated from a lizard captured from a poultry house in Nigeria(isolate 123nlf) were used as positive controls. The amplified products were resolved with precast E- gel in an Electrophoresis unit (Life Technologies).

**Analysis of the Quinolone resistant Determinant Region (QRDR) for the Levofloxacin resistant Salmonella isolate:** The MIC of the isolate to levofloxacin was determined by standard method according to the CLSI procedure (CLSI, 2009). The high fluoroquinolone resistant Salmonella isolate was subsequently screened for point mutation through the amplification of the gyrA of the quinolone resistant determining region and DNA sequencing of the PCR product. It was carried out as previously described (Ogunleye et al., 2011). A 560base pair region of gyrA of the crude boiled DNA was amplified with a universal forward and reverse oligonucleotide primers QRDR F=5´ATGAGCGACCTTGCGAGAAATACACCG3´ and QRDR R=5´TTCCATCAGCGCCCTTCAATGCTGATGTCTTC3´ using QS PCR reagents in a 50µl containing: 10 µl QS buffer, 1 µl dNTPs, 0.25 µl QRDF, 0.25 µl QRDR, 0.5 µl QS enzyme, 10 µl QS enhancer and 27 µl PCR water, using the PCR protocol: 98$^0$C for 30seconds, 35 cycles of 98$^0$C for 10seconds, 55$^0$C for 30seconds, 72$^0$C for 1minute 15seconds and 72$^0$C for 7minutes. One *Salmonella* Kentucky earlier isolated from septic poultry in Nigeria by Ogunleye and Carlson, 2012 was used as positive control. The amplified products were resolved with precast E- gel in an Electrophoresis unit (Life Technologies).

The amplified products were purified with Qiagens kits according to manufacturer's protocols and sequenced at Iowa State University DNA sequencing facilities (Ames, IA, USA).

## RESULTS

The isolate was identified as *Salmonella* enterica Give by National Veterinary Service Laboratories in Ames, Iowa State, USA. Figure 1 contain the oligo sequence of the synthesized Genscript$^R$ positive control. The isolate was positive for the sip C PCR amplification as shown in figure 2; Lane 1was loaded with DNA ladder; lane 4 showed the Sip C positive control *Salmonella* Kentucky earlier isolated from septic poultry in Nigeria while lanes 9-12 were showed the Sip C positive band of *Salmonella* Give isolate.

The *Salmonella* serotype was multidrug resistant: it was resistant to ceftazidime, ceftriaxone, amikacin, cefepime, levofloxacin, sulfamethoxazole, chloramphenicol, kanamycin, ampicillin and streptomycin at the respective breakpoint concentrations as shown in table 1. It has a very high MIC of >128µg/mL for levofloxacin. There was amplification of the gyrA of the quinolone resistant determining region of the isolate as shown in figure 3; lanes 1-2 showed the band for QRDR positive Salmonella Kentucky from septic poultry in Nigeria (MIC 64µg/mL), lanes 3-4 showed the bands for the QRDR positive Salmonella Give while lane 5 contained the DNA ladder.

The analysis of the sequencing of the amplified product shows that the QRDR positive *Salmonella* Give from lizard contains gyrA mutation encoding the histidine to tyrosine substitution at amino acid 150 (H150Y). The isolate did not bear any of the SGI1 integron genes as reflected in figure 4 where lanes 1-7 were loaded with integron negative Salmonella Give, whereas lanes 8-9 shows Integron positive *Salmonella* Kentucky isolated from septic poultry from Nigeria and lane 10 contained the DNA ladder.Figure 5 shows the result of the NDM-1 screening for the *Salmonella* Give isolate.

**Table 1:**
Antibiotic resistance pattern of the *Salmonella* Give from lizards

| isolate | ceftaz | ceftria | amik | cefep | Levo | Sulf | chloram | Kan | Amp | Strep |
|---|---|---|---|---|---|---|---|---|---|---|
| *Salmonella* Give | R | R | R | R | R | R | R | R | R | R |

*Key: Ceftaz=ceftazidime; ceftria= ceftriaxone; amik= amikacin; cefep= cefepime; levo=levofloxacin; sulf= sulfamethoxazole, kan= kanamycin; amp= ampicillin; strep= streptomycin; chloram= chloramphenicol; R=resistant*

ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCTGCATTGATGCT
GAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCAAATGGAAACTGGCGACCAACGG
TTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAATGTCTGGCAGCACACTTCCTATCTCGACATGCCG
GGTTTCGGGGCAGTCGCTTCCAACGGTTTGATCGTCAGGGATGGCGGCCGCGTGCTGGTGGTCGATAC
CGCCTGGACCGATGACCAGACCGCCCAGATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCG
CGCTGGCGGTGGTGACTCACGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGG
GATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAAC
ACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAG
GTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCACCGACATCGC
TTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTG
AGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGC
CATTCCGCCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCGACAAGCTGCGCTGA

**Figure 1:**
Oligosequence of the positive control by Genscript$^{R}$:

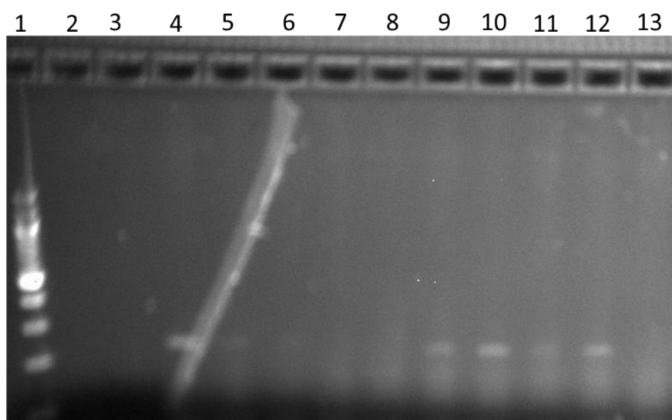


**Plate 1:**
Sip C screening for the *Salmonella* Give**.** Lane 1**,** DNA ladder; lane 4, Sip C positive control *Salmonella* Kentucky from septic poultry in Nigeria; lanes 9-12, Sip C positive *Salmonella* Give isolate

Lane 1, contained the DNA ladder; Lanes 2-11 were loaded with NDM-1 negative Pseudomonas aeruginosa isolated from lizard in Nigeria, lane 12 with an NDM-1 positive *Pseudomonas aeruginosa* from lizard (isolate 123nlf); lanes 13-15 contained the NDM-1 negative *Salmonella* Give; while lane 16 showed the band for Genscript$^{R}$ NDM-1 positive control.

## DISCUSSION

*Salmonella* enterica subsp. enterica serovar Give is an enterica serotype frequently isolated from ruminants and pigs but rarely found in human hosts (Haggin et al., 1997). However the serotype was isolated from a case of splenic abscess in Germany, it was recovered from a clinical case of an immunocompetent patient presented with splenic abscess due to S. ser. Give, which was associated with consumption of raw minced meat. (Girardin et al., 2006). It was also observed in Germany, that *Salmonella* Give meat associated infection resulted in a higher hospitalization compared with other non-Typhoidal serotypes like *Salmonella* ser Enteritidis, the observation was thought to be due to higher virulence of the serotype compared to other non-typhoidal serotypes (Girardin et al., 2006).

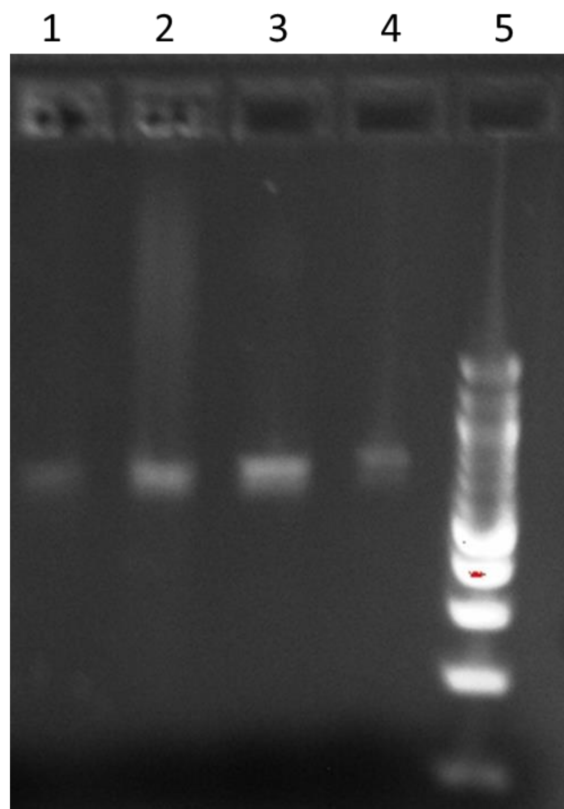


**Plate 2.**
Amplification of the quinolone resistant determining region (QRDR) of the *Salmonella* Give from lizard. lane 5, DNA ladder; lanes 3-4, QRDR positive *Salmonella* Give; lanes 1-2, QRDR positive *Salmonella* Kentucky from septic poultry in Nigeria (MIC 64µg/mL).

In this work a multidrug resistant *Salmonella* Give that was multidrug resistant, showing resistance to all the 10 antibiotics tested namely; ceftazidime, ceftriaxone, amikacin, cefepime, levofloxacin, sulfamethoxazole, chloramphenicol, kanamycin, ampicillin and streptomycin was isolated from the cloaca swab from a lizard captured in a poultry house in Nigeria. The resistance patterns observed in this isolate is unlike those of more susceptible *S.* Give isolates recovered from Dairy farms in Quebec where all were sensitive to ampicillin, apramycin, ceftiofur, cephalothin, enrofloxacin, gentamicin, neomycin, spectinomycin, tetracycline, and trimethoprim- sulfamethoxazole (Higgins et al., 1997).

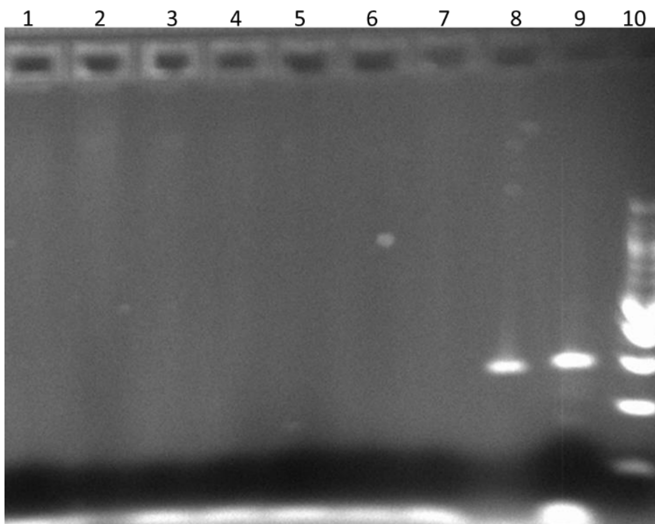


**Plate 3**
Integron screening for the Salmonella Give from Lizard. Lane 10, DNA ladder; lanes 1-7 integron negative *Salmonella* Give; lanes 8-9, Integron positive Salmonella Kentucky isolated from septic poultry from Nigeria.

The isolation of this serotype from lizard in poultry house is an indication of the public health risk associated with the presence of *Agama agama* lizards in poultry houses in terms of the possibilities of their role in transmission of Salmonella infections to poultry and human working in poultry houses. This becomes important because of the free access of lizards to poultry feed and poultry water sources in most commercial poultry in Nigeria. The *Salmonella* Give isolate although did not carry SGI1 gene nor NDM1 gene despite its resistance to ten antibiotics tested, it however carried point mutation with H150Y substitution at gyrA subunit of the quinolone resistant determining region. The possibility of the isolates carriage of some other plasmid borne resistant factors could not be ruled out since they were not tested for in this study. To the best of our knowledge, this work reports the isolation and characterization of *Salmonella* Give from lizard and therefore brings to light that lizards possesses a public health risk in terms of transmission of Salmonellosis to poultry and human in the study area. The multidrug resistance pattern of the Salmonella serotype also point to the possibilities of transference of drug resistant pathogen from lizard to food animals and human.

***Acknowledgement:***
*This work was funded by the grant provided by the Nigeria University Revitalisation fund, 2015 awarded by the University of Ibadan for a short Veterinary diagnostic Training and Research at the College of Veterinary Medicine, Iowa State University of Science and Technology, Ames, Iowa State, USA.*

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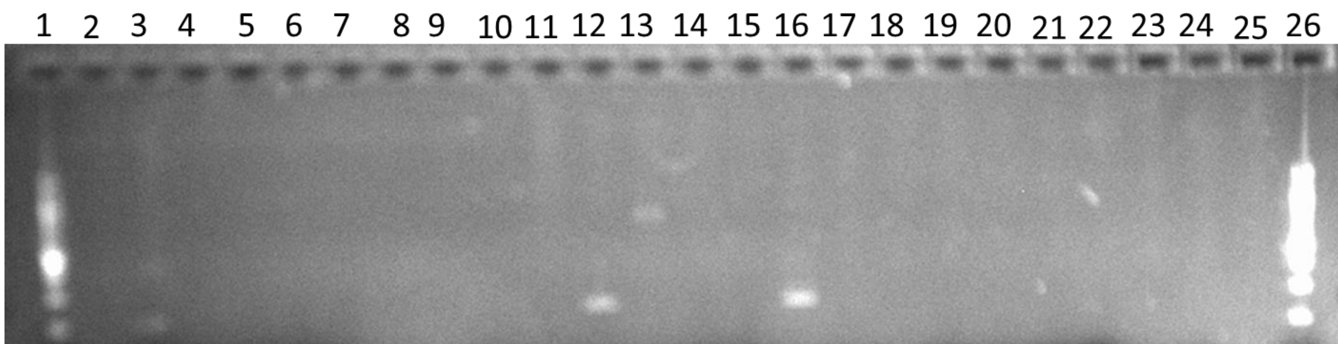


Plate 4.
NDM-1 screening for *Salmonella* Give isolate from lizard**. lane 1**, DNA ladder; lane 12, NDM-1 positive *Pseudomonas aeruginosa* from lizard (isolate 123nlf); lanes 13-15, NDM-1 negative *Salmonella* Give; lane 16, Genscript[R] NDM-1 positive control.

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