*Original Article*

# Emergence of an SGI1-bearing *Salmonella enterica* serotype Kentucky isolated from septic poultry in Nigeria

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## Abstract

Introduction: *Salmonella enterica* serotype Kentucky was isolated from septic poultry in Nigeria. The objective of this study was to characterize this isolate by screening for SGI1 and hyper-virulence.

Methodology: The strain was characterized by identification of *Salmonella* genomic island 1 (SGI1) through a PCR assay and we used a tissue culture invasion assay to assess protozoa-mediated hyper-invasion.

Results: *Salmonella* genomic island 1 (SGI1) was identified in the strain along with an SGI1 gene (SO13) implicated in hyper-virulence. Protozoa-mediated hyper-invasiveness was also documented in the strain.

Conclusion: The hyper-invasion is concordant with this emerging strain's ability to cause fowl paratyphoid.

**Key words:** *Salmonella enterica* serotype Kentucky; septic poultry; Nigeria

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## Introduction

Many strains of *Salmonella* are becoming resistant to multiple antibiotics and *Salmonella enterica* serotype Typhimurium phage type DT104 (DT104), a strain which is often resistant to five or more antibiotics, is a paradigm for this phenotype [1]. The DT104 antibiotic resistance genes are clustered within a 43-kb integrated element known as the *Salmonella* genomic island 1 (SGI1 [2,3]. The original SGI1 isolated from *Salmonella* serovar Typhimurium DT104 strains contained five antibiotic resistance genes encoding resistance to chloramphenicol and florfenicol (*floR*), ampicillin and carbenicillin (*blaP1*), streptomycin and spectinomycin (*aadA2*), sulfonamides (*sul1*), and tetracycline (*tetG*) [1,3,4]. SGI1 also contains approximately 30 genes that do not contribute to antibiotic resistance [3]. SGI1 has been reported in other phage types (e.g. DT120, DT193, U302) and in other serotypes including Agona, Albana, Indiana, Infantis, Kentucky, Meleagridis, Newport, Paratyphi, and Seftenberg.

DT104 bearing SGI1 were found to be hyper-invasive following exposure to rumen protozoa (RPz) that are normal flora of cattle [5]. In a subsequent study, an SGI1 gene (designated as SO13) gene was identified to coordinately up-regulate the observed hyper-invasion together with an unknown SGI1 gene(s) [6]. These studies accounted for the increased mortality of calves infected with DT104 when compared to calves infected with antibiotic-sensitive *S. enterica* serovar Typhimurium [7]. This finding is also true of other *Salmonella* serotypes, such as Agona and Infantis, possessing SGI1 [5].

Recently, we were apprised of an outbreak of systemic salmonellosis in poultry in Nigeria. Multi-resistant *Salmonella enterica* serotype Kentucky (*S.* Kentucky), showing antibiotics phenotypic characteristics typical of DT104, was isolated from this outbreak. We investigated the possibility that this multi-resistant *S.* Kentucky isolate contains SGI1 that is found in numerous serotypes [8,9-12] and is associated with hyper-virulence [5-7,13]. The current study characterizes the causative agent of the outbreak by screening for SGI1 and hyper-invasion. Our results identify a potentially emerging multi-resistant strain of *S.* Kentucky capable of causing significant systemic disease in poultry through a hyper-virulence mechanism.

## Methodology

*The* Salmonella *isolate and the study*

The *Salmonella* isolate used for this study was recently obtained from organs of septic poultry in commercial poultry farms in Nigeria. Birds exhibited clinical signs of septicemia characterized by depression, prostration, anorexia, pyrexia, and death [14].

*Serotyping of the isolate*

The multidrug resistant poultry isolates were sub-cultured into tripticase soy agar (TSA) and submitted to National Veterinary Service Laboratories in Ames, Iowa, USA, for serotyping. Serotyping was performed per the Kauffman-White Scheme.

*Determination of the minimum inhibitory concentrations of antibiotics*

Ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (all obtained from Sigma-Aldrich (Jos, Nigeria). Minimum inhibitory concentrations (MICs) were determined using the two-fold micro-broth dilution method per Clinical and Laboratory Standards Institute (CLSI) standards [15]. MICs were ascribed to the lowest concentration of the antibiotics that inhibited visible growth of the isolate while DT104 was used as the positive control.

*SGI1 screening and invasion assay*

Since the multi-resistance and putative hyper-virulence of the isolate were suggestive of the presence of SGI1, we examined the isolate for the presence of SGI1 elements such as SO13. The latter gene is involved in protozoal-mediated hyper-virulence of *Salmonella* through hyper expression of HilA while the former contains the following antibiotic resistance gene markers: *floR* (chloramphenicol/florfenicol resistance), *tetR* (regulator of tetracycline resistance), and *aadA2* (streptomycin/spectinomycin resistance) [6]. We used a forward primer specific to *floR* and a reverse primer specific to *tetR* for the antibiotic resistance genes *floR* (chloramphenicol/florfenicol resistance), *tetR* (regulator of tetracycline resistance), and *aadA2* (streptomycin/spectinomycin resistance) in a PCR assay as earlier described [6,16]. A PCR product corresponding to *aadA2* was purified and sequenced at the Iowa State University DNA Sequencing Facility (Ames, IA, USA).

As earlier described [6,16], approximately $10^9$ colony forming units of bacteria were added to approximately $10^5$ Rumen protozoa (RPz). The *Salmonella*-RPz mixture was then gently rolled for 16 hours at 37°C in a sealed 5-ml glass tube. Extracellular bacteria were then killed using 300 µg/ml florfenicol (Schering-Plough). For *in vitro* studies, RPz were lysed for 60 seconds at 4,800 rpm using 2.5 mM glass beads and a Mini-Bead beater (Biospec Products, Bartlesville, OK, USA). The lysate was centrifuged at 15,000 rpm for 2 minutes and then resuspended in 350 µl Lennox L broth. The lysate was used in a tissue culture invasion assay to assess protozoa-mediated hyper-invasion by allowing free-living protozoa (*Tetrahymena)* to engulf the strain prior to the HEp-2 cell tissue culture invasion. Percent invasion was calculated by dividing CFU recovered by CFU added [5,6].

## Results

Gross and histopathological lesions observed in the carcasses from which the strain was isolated included hepatomegaly, bronze discoloration of the liver (Figure 1), fibrinonecrotic typhlitis (Figure 2), and multifocal hepatic degeneration suggestive of a paratyphoid nodule (Figure 3).

Serotype analysis revealed that the isolate is *S. enterica* serotype Kentucky. Antibiotic susceptibility tests showed that the strain is resistant to ampicillin (MIC > 256 µg/mL), chloramphenicol (MIC = 128 µg/mL), sulfamethoxazole (MIC = 2,048 µg/mL), and tetracycline (MIC = 128 µg/mL). The isolate was sensitive to streptomycin with an MIC value < 16 µg/mL, whereas the positive control DT104 was resistant to all five antibiotics.

As shown in Figure 4, the *S*. Kentucky isolate contains SO13 and exhibits hyper-invasion by this gene in a manner similar to that observed with DT104. PCR studies revealed that *floR* and *tetR* are present and adjacent in the genome of the *S*. Kentucky isolate. Additionally, PCR revealed the presence of *aadA2* despite streptomycin susceptibility in the *S*. Kentucky isolate. As shown in Figure 5, the *S*. Kentucky *aadA2* contains a deletion of a thymidine at nucleotide 198 that is concordant with streptomycin susceptibility via a truncated AadA2 protein lacking the ability to adenylate and inactivate streptomycin (Genbank accession number: JN119849).

**Figure 1.** Gross pathology of septic chickens infected with SGI1-bearing *S.* Kentucky. Moderate enlargement of liver with an arrow highlighting marked bronze degeneration.

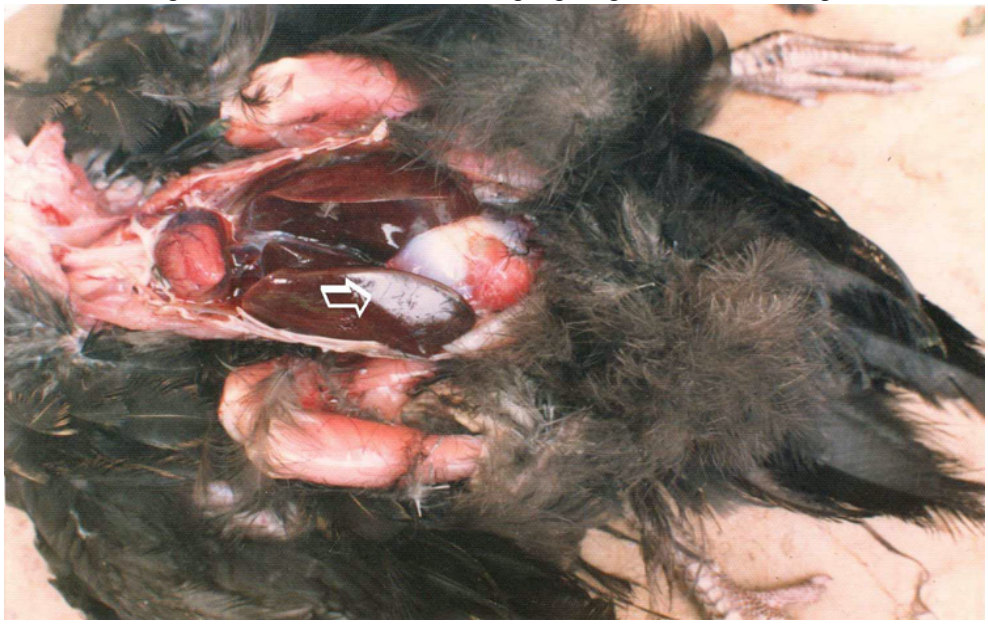


**Figure 2.** Gross pathology of septic chickens infected with SGI1-bearing *S.* Kentucky. The arrow indicates inspissation of fibrinonecrotic debris within the cecum.

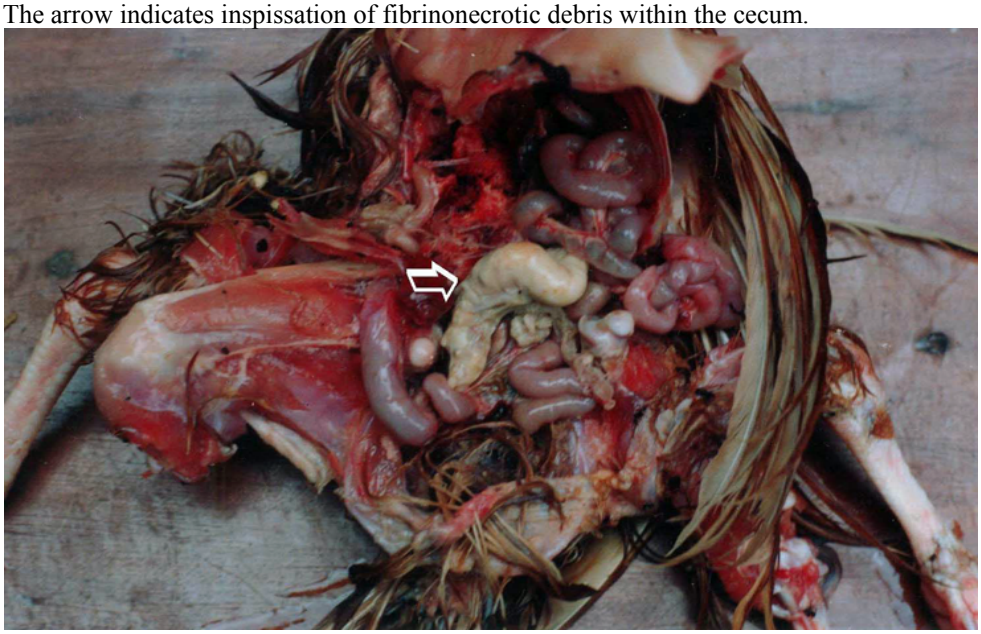
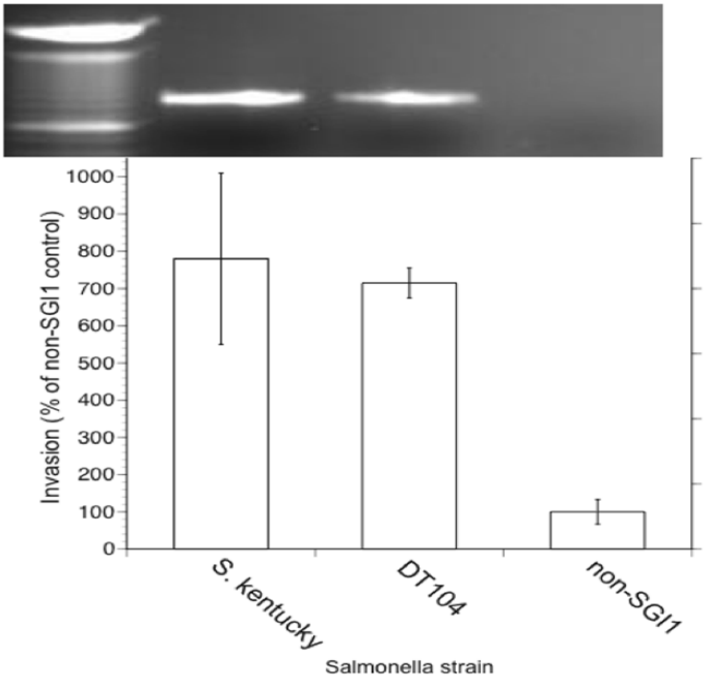


**Figure 3**. Histopathologic lesion of an apparent paratyphoid nodule in a septic chicken.

Arrows outline an area of hepatonecrosis and hyperplasia that is associated with systemic salmonellosis [18].

**Figure 4**. Hyperinvasion and the presence of SO13 in the *S*. Kentucky isolated from septic poultry.




DT104 was used as a positive control while SGI1-free *Salmonella* was used as a negative control. Invasion was measured in a tissue culture invasion assay following engulfment by and egress from *Tetrahymena* [5,6]. The gel picture insert above the invasion data illustrates the SO13 amplicons derived by PCR using primers described previously

**Figure 5.** Nucleotide and deduced amino acid sequence alignments for *aadA2* and AadA2, respectively, in SGI1-bearing *S.* Kentucky and *S.* Typhimurium DT104 (DT104).

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S. Kentucky   171GCTTGATGAAACGACGCGGCGAGCAT-GCTCAATGACCTTATGGAG…
DT104         171GCTTGATGAAACGACGCGGCGAGCATTGCTCAATGACCTTATGGAG…

S. Kentucky   57KLDETTRRACSMTLWRLRLSLARARRSAL*
DT104         57KLDETTRRALLNDLMEASAFPGESETLRAIEVTLVVHDDIIPWRYPA…
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A thymidine at nucleotide 198 is absent in *S.* Kentucky and this deletion encodes divergences at amino acid 66 and a truncation after amino acid 85 (gray underlined). (a) nucleotide sequence and (b) amino acid sequence.

## Discussion

Our study describes the characterization of *Salmonella enterica* serotype Kentucky isolated from septic poultry in Nigeria. This isolate contains SGI1 or an SGI1-like integron based on the presence of the SO13, *floR*, *tetR*, and *aadA2*. This isolate bears an *aadA2* isoform that does not confer streptomycin resistance due to an inactivating mutation in which the nucleotide 200 is missing and a different amino acid sequence starting at 66 (C instead of L) with a premature stop codon at amino acid 147 when compared with DT104. This observation is responsible for the sensitivity of this particular isolate to streptomycin, whereas DT104 is resistant.

Our previous studies have implicated protozoa as instigators of hyper-virulence in SGI1-bearing non-typhoidal [5,6] and paratyphoidal *Salmonella* [17]. DT104 may be more virulent than other *Salmonella* strains, and this putative phenomenon has been attributed to the presence of SGI1 [5]. Humans infected with DT104 are known to be two to three times more likely to be hospitalized than those infected with other strains [13], and calves infected with DT104 are 13 times more likely to die than calves infected with antibiotic-sensitive *S. enterica* serovar Typhimurium [7]. This finding is also extended to other *Salmonella* serotypes, *e.g.*, Agona and Infantis, which possess SGI1 [5]. (RPz, microbiota that natively inhabit the major forestomach of ruminants such as cattle, were identified as mediators of DT104 hyper-invasion. In a model study, RPz engulf DT104 and then hyper-activate the enteroinvasive phenotype. The RPz/DT104 bacteria are then moved to the abomasum, the "true" stomach of ruminants, where the RPz are digested and DT104 is released. DT104 then moves to the small intestine where invasion, or in this case hyper-invasion, ensues. Hyper-invasion leads to a faster onset of clinical signs, a greater recovery rate of the pathogen, and a poorer prognosis. This phenomenon was not observed in the absence of RPz or SGI1 [5]. Thus RPz and SGI1 appear to co-contribute to the enhancement of invasion.

In this study, *S.* Kentucky strain possesses DT104 like characteristics such as SGI1, therefore we assessed protozoa-mediated hyper-invasion in the isolate. *Tetrahymena*, a common free-living water-borne non-pathogenic protozoan, induced a significant increase in tissue culture invasion for *S.* Kentucky. This finding is thus consistent with the systemic nature of this isolate. Future studies will identify the specific protozoan from the affected poultry farms that possibly contributed to the hyper virulence / hyperinvasiveseness of this isolate. Nonetheless, this is the first report of an SGI1-bearing *Salmonella* isolate capable of causing avian paratyphoid from Nigeria.

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