

# INVA GENE AND ANTIBIOTIC SUSCEPTIBILITY OF SALMONELLA SPP ISOLATED FROM COMMERCIALY PROCESSED BROILER CARCASSES IN LUSAKA DISTRICT, ZAMBIA

## Research Article

TM Shamaila,<sup>1,2</sup> K Ndashe,<sup>3,4</sup> C Kasase,<sup>2</sup> M Mubanga,<sup>5</sup> L Moonga,<sup>5</sup> J Mwansa,<sup>4</sup> BM Hang'ombe<sup>5</sup>

1. Ministry of Fisheries and Livestock, P.O Box 38111, Monze, Zambia
2. Department of Public Health, School of Health Sciences, University of Lusaka, Lusaka, Zambia.
3. Department of Environmental Health, Faculty of Health Science, Lusaka Apex Medical University, Kasama Road, Lusaka, Zambia.
4. Department of Microbiology, Faculty of Medicine and Surgery, Lusaka Apex Medical University, Kasama Road, Lusaka, Zambia.
5. Department of Para clinical Studies, School of Veterinary Medicine, The University of Zambia, PO Box 32379, Lusaka, Zambia.

**Correspondence:** Kunda Ndashe (ndashe.kunda@gmail.com)

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*Poultry meat is considered to be one of the major vehicles of Salmonella infections in humans and has been implicated in outbreaks of Salmonellosis in humans. The potential for mass outbreaks of Salmonellosis is likely to be linked to the high consumption of poultry meat and the increasing antibiotic resistance of Salmonella spp poses a huge challenge to treatment of the foodborne infection. Determination of virulence genes such as InvA is important as they play a vital role in the establishment and invasion of Salmonella spp in the gastrointestinal tract of the host and therefore is means of detection of the virulence of the pathogen and it is the international standard in the detection of Salmonella spp using molecular techniques. This was a cross-sectional study where two abattoirs were sampled conveniently in Lusaka district. A total of 100 swabs were collected from the 2 abattoirs and submitted to the University of Zambia, School of Veterinary Medicine, Paraclinical Sciences laboratory where isolation and identification bacteria was conducted. Presumptive Salmonella colonies were further analysed through conducting biochemical tests, molecular detection of the virulence gene invA through polymerase chain reaction and antibiotic susceptibility testing.*

*The study revealed that 2% of commercially broiler carcasses were contaminated with Salmonella spp. The isolates further showed resistance to two antibiotics, gentamicin and tetracycline after antimicrobial susceptibility*

*testing. The presence of Salmonella spp with a virulence gene (InvA) in commercially processed broilers is a public health concern mostly in sensitive population and multi-drug resistance of the pathogen presents challenge in treatment options of Salmonellosis.*

## Introduction

Salmonellosis is a major public health concern and cases have been attributed to poultry and poultry products [1]. Contamination of poultry can occur through the whole production chain, from the farm to the abattoir [2]. Reports on the contamination of the broiler carcasses in abattoirs and in retail shops have been published by various workers [3-5] and among other reasons it is due to inappropriate handling and hygiene conditions [6]. Transport in inadequately cleaned and disinfected containers [7], cross-contamination by the slaughter environment or by Salmonella-contaminated flocks to the carcasses of Salmonella-free flocks are identified as possible risk factors in the abattoir [8, 9]. At slaughter, the gastrointestinal tract may harbour Salmonella and may be damaged during the evisceration, resulting in contamination of poultry carcasses [10]. For human two main pathways of exposure to salmonella in poultry meat have been identified and these are undercooking and cross-contamination [11]. During the cooking procedure, fairly high temperatures occur on the outside of meat but may not be sufficient

to kill bacterial pathogens inside [12]. The contaminated undercooked poultry meat is then associated with bacterial pathogens located inside the chicken meat and serves as an exposure pathway humans [12]. The constant use of antimicrobial agents in poultry production for palliative and curative purposes as well as growth promotion is contributing factor to the emergence of antibiotic resistant bacteria that are subsequently transferred to humans through the consumption of undercooked poultry meat [13]. The use of antibiotics as growth promoter has been banned in many countries globally but antimicrobial use regulation is still a challenge in many developing countries [13]. Misuse of antimicrobials is facilitated in developing countries by their availability over the counter, without prescription and through unregulated supply chains [14].

There are over 2500 serovars of Salmonella, but only a few are commonly associated with human disease [15]. The pathogenic process of salmonellosis is usually dictated by an number of virulence genes that act in tandem and ultimately manifest in the typical symptoms of salmonellosis [16]. The virulence genes are encoded within the Salmonella pathogenicity islands (SPI) on the chromosome as units of large cassettes and these genes encode products that assist the organism

in expressing its virulence in the host cells [17]. These products are produced as effector proteins and include *invA*, *sipA*, *sipB*, *sipC*, *sifA*, *hilA*, *hilC*, *hilD* and *invF* [18]. The distribution of these genes among the various isolates obtained from biological sources may be important in indicating the clinical significance of *Salmonella* [19]. The *invA* gene of *Salmonella* contains sequences unique to this genus and has been proved to be a suitable PCR target with potential diagnostic application [20]. This gene is recognized as an international standard for detection of *Salmonella* genus [21]. The *invA* gene plays a vital role in the establishment and invasion of *Salmonella* spp in the gastrointestinal tract of the host and therefore its detection serves as a means of determination of the virulence of the pathogen [22].

Zambia has recorded an increase in annual poultry production from 13 to 76 million broiler chickens between the 1990's to 2016 [23]. The growth of the poultry industry has seen an increase in the source of proteins for the local population and for export to neighbouring countries [24]. The financial potential of the fast expanding poultry industry has provided opportunities for establishment of commercial abattoirs where chickens are slaughtered, processed and packaged for the retail market. Owing to processing and packaging of poultry for the market, it is important that the meat is superior in quality and free of *Salmonella* contamination to ensure that the consumers are protected from any possible foodborne infection [25]. It was therefore imperative that a study was conducted on *Salmonella* spp in commercially processed broiler carcasses to determine its prevalence, antibiotic susceptibility and presence of the virulence gene (*invA*).

## Materials And Methods

This was a cross-sectional study conducted in Lusaka district, Zambia. The district has five commercial semi-automated poultry abattoirs of which only two (A and B) granted

permission to conduct the study from their premises. The chickens processed at the two abattoirs are distributed in supermarkets and sold to consumers countrywide.

One hundred (100) samples were collected, 50 from each abattoir. The two abattoirs were sampled conveniently and swabs were collected using systematic random sampling method. Every 25th processed broiler carcass was swabbed on the visceral and cloacal surfaces using the one swab, this was done just before being packaged and consequently taken for refrigeration. The cloacal and visceral surfaces were swabbed because they have been reported to harbour more gastrointestinal contents in cases of contaminations [26]. Each swab was then placed in test tube with selenite broth and stored in a cooler box before being transported to the laboratory.

The swabs collected in selenite broth were incubated for 24hours at 37°C after which they were cultured on Blood agar (Himedia, India), Maconkey agar (Himedia, India) and xylose lysine deoxycholate (XLD) agar (Himedia, India) at the same incubation conditions. Gram staining was performed on the colonies from XLD and Maconkey agars and bacteria that were identified as presumptive *Salmonella* spp was further inoculated Triple sugar iron agar (TSI) agar (Oxoid, UK) and SIM agar (Oxoid, UK), which were incubated at 37°C for 18hours. Other biochemical tests for the salmonella spp isolates included; Urease, citrate and Methyl Red Voges Proskauer (MRVP) media (Oxoid, UK).

The colonies identified as salmonella spp were then subjected to serotyping by slide agglutination, according to the Kaufmann-white classification using salmonella polyvalent O and H antisera (Denka Seiken co., Tokyo, Japan).

DNA from the enriched culture was extracted by heating for 10-20min on a heating block. The DNA was used as the

template for the PCR assay and the primers pair used targeted the *invA* gene and are shown below;

22 mer Sal141- 5'

TCATCGCACCGTCAAAGGAACC 3'

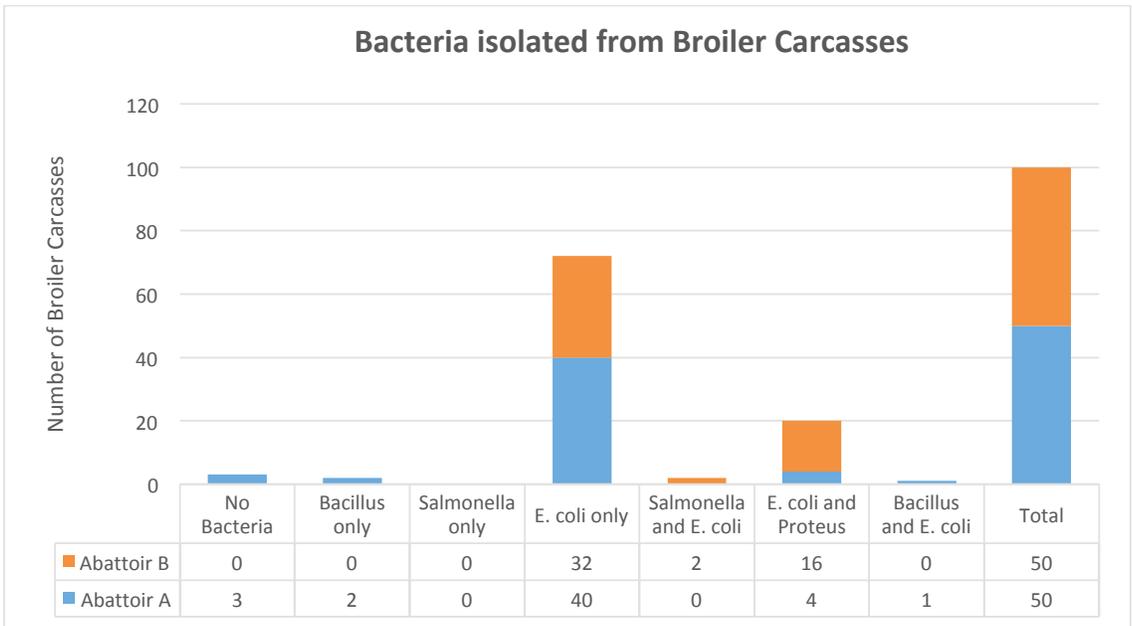
26 mer Sal139 -5'

GTGAAATTATCGCCACGTTCCGGCAA 3'

The total mixture for each sample came up to 25µl (24µl of master mix+ 1µl DNA template). The Master mix was prepared as follows;(a) 10× Buffer (2.5µl), (b) dNTP (2.0µl), (c) Primer (forward and reverse) 1.0µl, (d) Distilled water (18.377µl), (e) Enzyme Ex Taq (0.125µl)

Positive and negative test samples were run alongside the template DNA samples. The reaction mixture were run at the following conditions; denaturation at 94°C for 2 min.; thirty-five cycles of amplification at 95°C for 30 seconds, at 60°C for 30 seconds, and at 72°C for 30 seconds and the reaction was completed by a final 10 minutes extension at 72°C. Aliquots of amplification products were separated on 0.5% agarose gel in 0.5X Tris Borate Ethylene diamine tetra acetic acid (EDTA) buffer and visualized by ethidium bromide staining and UV transillumination

The antimicrobial susceptibility testing was done using the Kirby-Bauer disc diffusion method on Mueller Hinton Agar (Becton, Dickinson and Company, MD, USA) based on the Clinical Laboratory Standard Institute (CLSI) guidelines [27]. The antibiotic discs (Becton, Dickinson and Company, MD, USA) used included Nitrofurantoin (300µg), Cephoxitin (30µg), Kanamycin (30µg), Doxycycline (30 µg), Co-trimoxazole (1.25/23.75 µg), Amoxicillin and Clavulanic acid (AMC) (20µg/10µg), Tetracycline (30µg), Gentamycin (10µg), Chloramphenicol (30µg), and Oxacillin (1µg).



**Figure2: Number of broiler carcasses contaminated with bacteria**

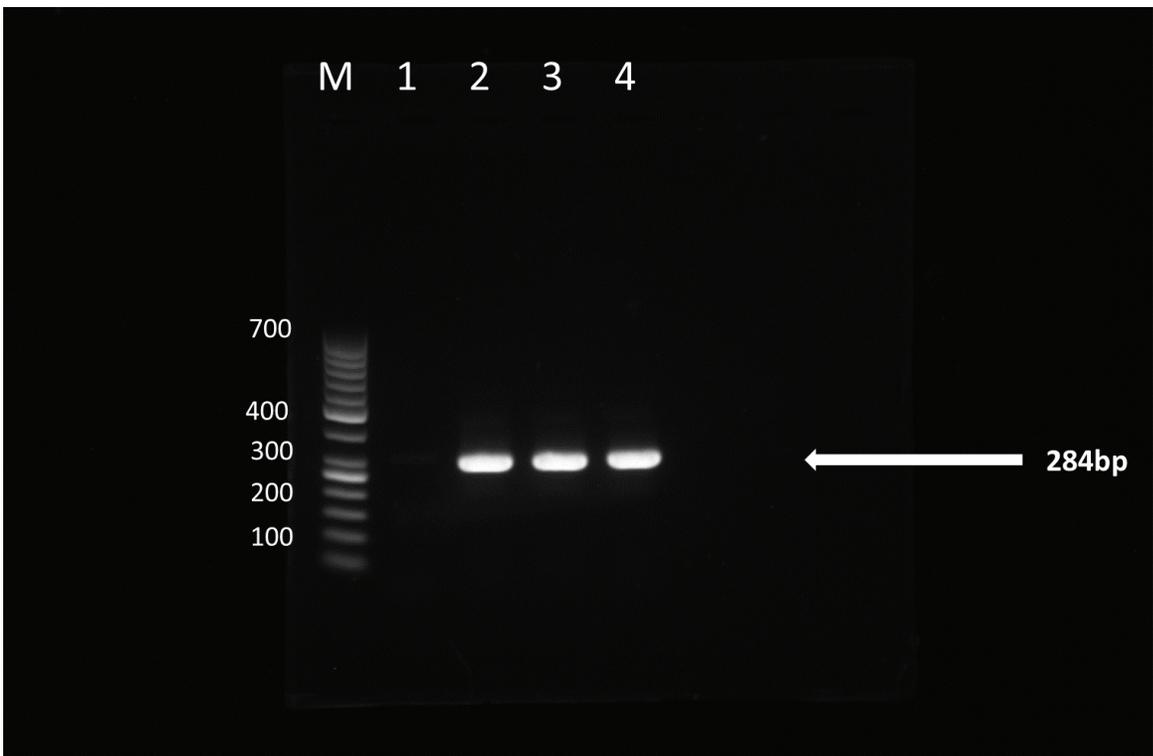


Figure 2: PCR detection of *Salmonella virulent gene invA*, Lane 1 to 4. M, marker with 50 bp ladder, with amplicons being indicated by the arrow. Lane 1 and 2 being the negative and positive controls, respectively, Lane 3 and 4 are the *Salmonella* isolates of broiler carcasses #76 and #94.

**Table 2: Antimicrobial sensitivity for Salmonella isolates from broiler carcasses**

S/N	Antibiotic	Zone Diameter Interpretive Criteria (nearest whole mm)*			Size of inhibition Zone (mm)	Comment
		Susceptible	Intermediate	Resistant		
1	Nitrofurantoin (300µg)	≥17	15 to 16	≤14	19	Susceptible
2	Cephoxitin (30µg)	≥18	15 to 17	≤14	17	intermediate Susceptible
3	Kanamycin (30µg)	≥18	14 to 17	≤13	17	intermediate Susceptible
4	Doxycycline (30 µg)	≥14	11 to 13	≤10	27.5	Susceptible
5	Co-trimoxazole (1.25/23.75µg)	≥16	11 to 15	≤10	20	Susceptible
6	Amoxicillin and Clavulanic acid(AMC) (20µg/10µg)	≥18	14 to 17	≤13	30	Susceptible
7	Tetracycline (30µg)	≥15	12 to 14	≤11	10	resistant
8	Gentamicin (10µg)	≥15	13 to 14	≤12	11	resistant
9	Chloramphenicol (30µg)	≥18	13 to 17	≤12	17	Partially Susceptible
10	Oxacillin (1µg)	≥13	11 to 12	≤13	13	Susceptible

*\*Values obtained from the Clinical Laboratories Standards Institute (CLSI) Guidelines for Performance Standards for Antimicrobial Susceptibility Testing.*

## Results

### a. Bacteria isolated from Broiler carcasses

A total of 100 swabs were collected from abattoir A and abattoir B, 50% coming from each site. The results of the study revealed that only 2 broiler carcasses were contaminated with *Salmonella* spp. Other bacteria that were isolated from the broiler carcasses included *Escherichia coli* (*E. coli*), *Proteus* spp, and *Bacillus* spp [table 1]. The most prevalent bacteria was *E. coli* which was isolated from 72 broiler carcasses, and it coexisted with other microorganisms such as *Proteus* spp, *Salmonella* spp and *Bacillus* spp [Table 1].

### b. Molecular detection of *Salmonella* spp

The two *Salmonella* isolates that were subjected to PCR with primers that targeted the *invA* gene revealed amplicons with a band size of 284bp and were identified using gel electrophoresis [Figure 1]. This results therefore demonstrated the presence of *invA* gene in the *Salmonella* spp that was isolated from broiler carcasses.

### c. Antibiotic sensitivity test of *Salmonella enteritidis*

The two *Salmonella* isolates were susceptible to Nitrofurantoin, Doxycycline, Co-trimoxazole, Amoxicillin and Clavulanic acid and Oxacillin. The isolates showed partial susceptibility to Cephoxitin, kanamycin and chloramphenicol. Resistance was observed on tetracycline and gentamicin.

## Discussion

From the study *Salmonella* was isolated from only 2 broiler carcasses (2%), the *invA* gene was detected from the isolates and they were resistant to tetracycline and gentamicin. In previous studies conducted in Lusaka District, the prevalence of *Salmonella* in broiler carcasses ranged from 20.53% to 46.15%, from 1998 to 2012, respectively [24, 28-29]. In a study by Hang'ombe et al (1998) a prevalence of 28% of *Salmonella* in processed broiler carcasses was reported, of which 16.82% was *Salmonella enteritidis* [28] and in another study the prevalence of *Salmonella enteritidis* was reported at 20.53% [29]. The previous studies sampled

both commercially and backyard processed broiler carcasses, therefore comparison of the prevalence is insignificant since the present study sampled commercially processed broiler carcasses only. The low prevalence of *Salmonella* in the study could be due to hygiene standards in commercial poultry abattoirs. Abattoirs in Lusaka adhere to strict Hazard Analysis Critical Control Point (HACCP) standards throughout the production process, thereby ensuring low microbiological contamination of broiler carcasses [30]. It was further reported that routine *Salmonella* surveillance activities are conducted by Department of Veterinary Services (DVS) through Central Veterinary Research Institute of Zambia (CVRI) to ensure high standards are upheld and poultry meat sold to public is free of *Salmonella* [31].

The *invA* gene which codes for proteins which are necessary for invasion of epithelial cells was present in the *Salmonella* isolated from commercially broiler carcasses. The isolation of invasive *Salmonella* serotypes from broiler carcasses in Lusaka district poses a public health risk as contaminated chicken serves as vehicle for transmission when consumed undercooked. Non-typhoidal salmonellosis (NTS) infections are clinically present as self-limiting gastroenteritis [32] but bacteraemia and other complications have been reported in sensitive populations (geriatrics, paediatrics, and immunocompromised individuals) [33, 34]. It is therefore important that poultry abattoirs produce broiler carcasses that are free of *Salmonella*.

Antibiotic sensitivity test results revealed that the *Salmonella* isolated from broiler carcasses were resistant to tetracycline and gentamicin. In study by Ulaya et al (2012), it was reported that *Salmonella* isolated from chickens showed higher resistance to tetracycline, gentamicin, vancomycin, erythromycin and co-trimoxazole [35]. Other bacteria isolated from poultry have also demonstrated multiple antibiotic resistance. In a study by Chishimba et

al (2016), they reported high resistance rates of *Escherichia coli* isolated from poultry to ampicillin (100%), cefotaxime/ceftazidime (100%), tetracycline (59.7%), chloramphenicol (57.1%), and norfloxacin (54.5%) [36]. Tetracycline and gentamicin are extensively used in the poultry industry for treatment of a variety of diseases and also as growth promoters. The continued misuse of tetracycline and gentamicin in rearing of broilers has contributed to the development of resistant bacterial strains [37]. The resistance pattern observed with the *Salmonella* isolates in the study raises concern on the increasing antibiotic resistance of bacteria isolated from poultry.

## Conclusion

The study confirmed a prevalence of 2% of a virulent multidrug resistant *Salmonella* spp in commercially processed broiler carcasses and this raises concerns on the public health risk to consumers of chickens. Furthermore, the study confirmed the resistance to tetracycline and gentamicin, which are commonly used antibiotics in poultry production.

## Recommendations

The authors of the study therefore recommend that the Government of Republic of Zambia (GRZ) through the Zambia Medicines Regulatory Authority (ZAMRA) should institute controls on the indiscriminate purchase of antibiotics for livestock production. The on-counter purchase of antibiotics by poultry producers has led to the abuse of the drugs which are reportedly used in none bacterial infections and are often administered at lower doses than recommended. Furthermore, the authors propose the creation of an agency that will investigate the increasing trends of antibiotic resistance in both human and veterinary medicine. *The Department of Veterinary Services through the Central Veterinary Research Institute should continue with the routine Salmonella inspections to monitor the HACCP systems that are established in all the poultry abattoirs in Zambia.*

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