Detection of *invA*, *spiC*, *sipC*, *invF*, and *hilA* in *Salmonella* Isolated From Beef and Poultry by Dot Blot Hybridization in Zambia

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ABSTRACT

A *Salmonella* organism is one of the most important food-borne zoonoses in the world. This study illustrates the application of the dot blot hybridization assay to identify *Salmonella* strains isolated from eggs, chicken, and beef carcasses that possess virulence determinants that have been identified as significant factors in pathogenesis of the organism over the years. All the 59 *Salmonella* isolates examined were found to possess *spiC* and *invA* genes essential for *Salmonella* spp. to enter intestinal epithelial cells. Other effector proteins were also found in the order of *sipC* (66.1%), *invF* (37.3%), and *hilA* (11.9%). The presence of these virulence determinants clearly demonstrates the significance of dot blot hybridization assay to differentiate *Salmonella* strains in the absence of molecular typing tools.

INTRODUCTION

Salmonella enterica is a facultative intracellular pathogen that is capable of causing disease in a range of hosts. In poultry, it can occur as a severe systemic disease responsible for heavy economic losses to the commercial poultry industry through morbidity, mortality, and reduced egg production.[1,2] It is also one of the major food-borne causes of gastroenteritis, often associated with contaminated meat, especially poultry meat.[1] In Zambia, the detection of *Salmonella* spp. in chickens and eggs is fully reported.[3,4]

Salmonellosis in Zambia is usually detected through a combination of serologic tests and bacteriological confirmation combined by biochemical reactions, especially in the differentiation of *Salmonella enteritidis*

from the non-motile, closely related strains of *Salmonella pullorum/gallinarum*. The bacteriological methods of identifying *Salmonella* through culturing are labor-intensive, expensive, and time-consuming. They include selective enrichment and plating followed by biochemical tests.[5] This technique cannot indicate the important characteristics of the isolates regarding genetic determinants of causing disease.

It has been documented that polymerase chain reaction (PCR) for the specific detection of *Salmonella* is evaluated targeting an *invA* gene, which is a marker gene.[6,7] The *invA* gene codes for protein in the inner membrane of bacteria that is necessary for invasion of epithelial cells.[8] Recently, many factors and a complex of mechanisms are being unfolded regarding the expression of *Salmonella* virulence. Most of the virulence genes are encoded within *Salmonella* pathogenicity islands (SPIs) on the chromosome as units of large cassettes and some operons.[9] One of the most important SPI functions encoded on the chromosome is for passage across the intestinal epithelium by invading the M cells in Peyer's patches of the intestines. SPI1 encodes the type III secretion apparatus with several effector/regulatory proteins that modify host cell signaling pathways. Some of these secreted effector proteins include *sipA*, *sipB*, *sipC*, *sifA*, *hilA*, *hilC*, *hilD*, and *invF*.[10-13] These are clusters of chromosomal virulence genes found only within the genus *Salmonella*.[14] Various *Salmonella* serovars show the same sequences upon PubMed protein and genome sequence search. *Salmonella* bacteria containing mutations in these genes have shown to be attenuated in mice infected orally but fully virulent after intraperitoneal administration, presumably because of their inability to gain access to the intestinal epithelium.[15] It is very clear that most of the *Salmonella* infections are as a result of food ingestion. In this study, we tried dot blot hybridization to screen *hilA*, *spiC*, *sipC*, *invF*, and *invA* genes from *Salmonella* isolated from beef and poultry sources. Such elucidation of the distribution of these SPI genes in *Salmonella* serovars may yield a better understanding on the occurrence of pathogenic serovars in Zambia.

MATERIALS AND METHODS

The chicken and cattle carcasses were sampled from various markets in Lusaka. *Salmonella* was isolated from rinse solutions. Twenty-five grams were sampled aseptically and rinsed in 50-mL phosphate buffer solution. The rinse solution (1 mL) was incubated in the selenite broth for 18 hours at 37°C. In case of *Salmonella* isolation from eggs, 1 mL of the egg contents was inoculated in 10 mL of selenite broth and incubated for 18 hours at 37°C. This was followed by culturing directly on *Salmonella Shigella* (SS) agar for 24 hours at 37°C. The identity of suspected *Salmonella* isolates was confirmed by standard methods.

For whole cell extracts preparation, the isolated *Salmonella* was incubated in brain heart infusion (BHI) broth overnight. A culture of hemolytic *Escherichia coli* isolated from a clinical case was grown as a negative control. This was done to clearly show the existence or non-existence of the effector proteins under specific study for *Salmonella*. Following incubation, 1 mL of the BHI broth was centrifuged at 3000 rpm for 10 minutes to harvest bacterial cells. After centrifugation, the pellet was treated with 100 µL of SDS buffer (1% SDS in 50 mM Tris-HCl, pH 8.0). The mixture was heated at 95°C for 3 minutes and then preceded to do the dot blot hybridization assay using the whole cell extracts as the antigen. The whole cell extracts was dot spotted on the nitrocellulose membrane (Schleicher & Schuell Bioscience, Dassel, Germany) by using a copy steel plate capable of transferring 25 µL of sample. The copy steel plate transfers 48 samples at a time. After transferring the antigen extracts on to the membranes, the membranes were allowed to dry at room temperature, and then blocking with 5% skim milk in PBS for 1 hour at room temperature was performed. After washing 5 times in 30 minutes with PBS, the membranes were incubated for 1 hour

at room temperature with the first rabbit antibody against *sipC*, *spiC*, *invA*, *invF*, and *hilA* (1:2,500 dilution) for each respective membrane in 0.5% skim milk. The membranes were washed and treated with goat anti-rabbit antibody (1:5,000 dilution) for 1 hour at room temperature. After washing the membranes were developed in 5-mL TMB (tetramethyl-benzidene) stabilized substrate for horseradish peroxidase (Promega Co., Madison, Wisconsin). To stop the reaction, the membranes were rinsed in distilled water and then air-dried.

Isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) and chromatostrip detection of *Salmonella* was performed by using Takara ICAN (*Salmonella* detection kit-chromatostrip version, Takara Bio Co., Ltd, Kyoto, Japan) as described by Isogai et al.[16]

RESULTS

We examined 59 samples of *Salmonella* isolated from eggs, chicken, and beef carcasses (Table 1) for the presence of effector proteins and the *invA* gene by dot blot hybridization. The basis of determining positives in the membranes is shown in Figure 1. Only membranes for *invA*, *spiC*, and *sipC* are shown. As can be clearly seen, the *invA* and *spiC* showed a 100% positive for the given membranes. These results demonstrate a clear abundance of *invA* and *spiC* in the analyzed *Salmonella* strains. On ICAN, all the *Salmonella* strains examined were positive for *Salmonella invA*. All the 59 isolates were positive to *Salmonella invA* when detected by ICAN. All the dots positive to each effector protein are quite distinct (Figure 1).

All the examined 59 isolates showed the presence of *invA* and *spiC* (100%) while 39 (66.1%) isolates were positive for *sipC*, 7 (11.9%) for *hilA*, and 22 (37.3%) positive for *invF* (Figure 2). The isolates from beef carcasses were all positive for *invA*, *spiC*, and *sipC*. Out of 13 beef isolates, 4 (30.8%) were positive for *hilA* and 9 (69.2%) for *invF* (Figure 3). Only 3 beef isolates were positive to all determinants being investigated (*invA*, *sipC*, *spiC*, *hilA*, and *invF*). The eggs and chicken carcasses isolates had 26 (56.5%) isolates positive for *sipC*, 3 (6.52%) isolates for *hilA*, and only 13 (28.3%) were positive for *invF* (Figure 4). Only 2 isolates from chicken carcasses were positive to all determinants under investigation in this study, whilst all isolates from the eggs were negative for *hilA*. On a percentage basis, the isolates from beef had a higher percentage of positives for *hilA* (30.8%) and *invF* (69.2%) despite the low number of isolates being used for comparison purposes.

Table 1. Source of *Salmonella* used in the dot blot hybridization assay.

***Salmonella* Source**	***Salmonella* Isolates, n**
Beef carcasses	13
Chicken carcasses	27
Eggs	19
Total	59

DISCUSSION

The goal of this study was to determine whether *Salmonella* effector proteins, which are secreted across the inner and outer membranes of the bacterial cell, are found in strains isolated from beef and chicken sources. It has been documented that effector proteins contribute to *Salmonella enterica* invasion of epithelial cells.[10] Upon secretion from the bacterial cell, these proteins are thought to form a complex in the eukaryotic membrane that is required for translocation of other effectors into the host cell cytoplasm triggering the formation of ruffles in the host cell membrane resulting in bacterial internalization.[17,18] It is quite clear that one of the major routes of *Salmonella* infection is by ingestion, hence our result being 100% positive to *invA*, which is necessary for invasion of epithelial cells.[8]

Isolation of *Salmonella* with these effector proteins from food origin clearly shows the preparedness of these strains to propagate across the intestinal epithelium. Furthermore, all the 59 isolates were positive for the presence of *spiC* protein, which interferes with intracellular trafficking and is required for translocation of *Salmonella*

Figure 1. Dot blot hybridization for the detection of *invA*, *spiC*, and *sipC* in various *Salmonella* strains. Whole cell extracts were prepared from various *Salmonella* strains and then dot spotted on the nitrocellulose membranes. The membranes were immunostained with specific antibody against anti-*invA* (A), anti-*spiC* (B), and anti-*sipC* (C).

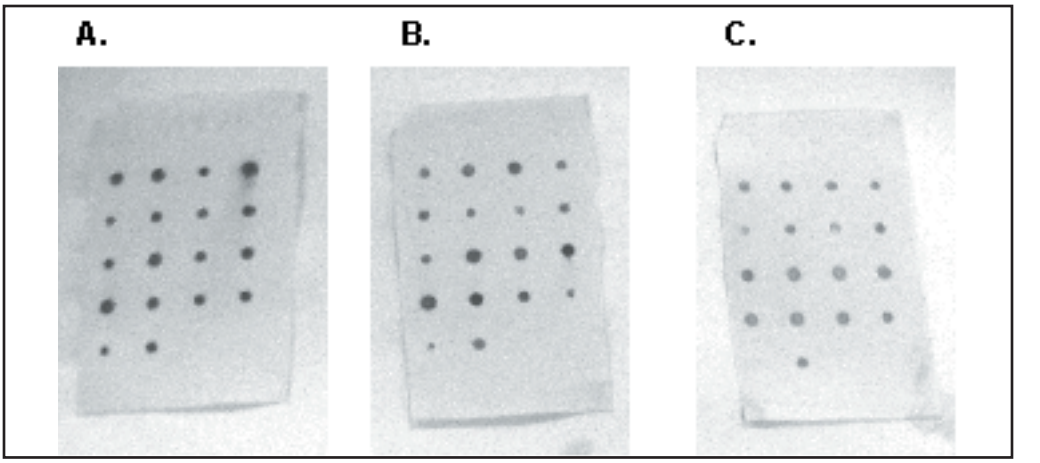
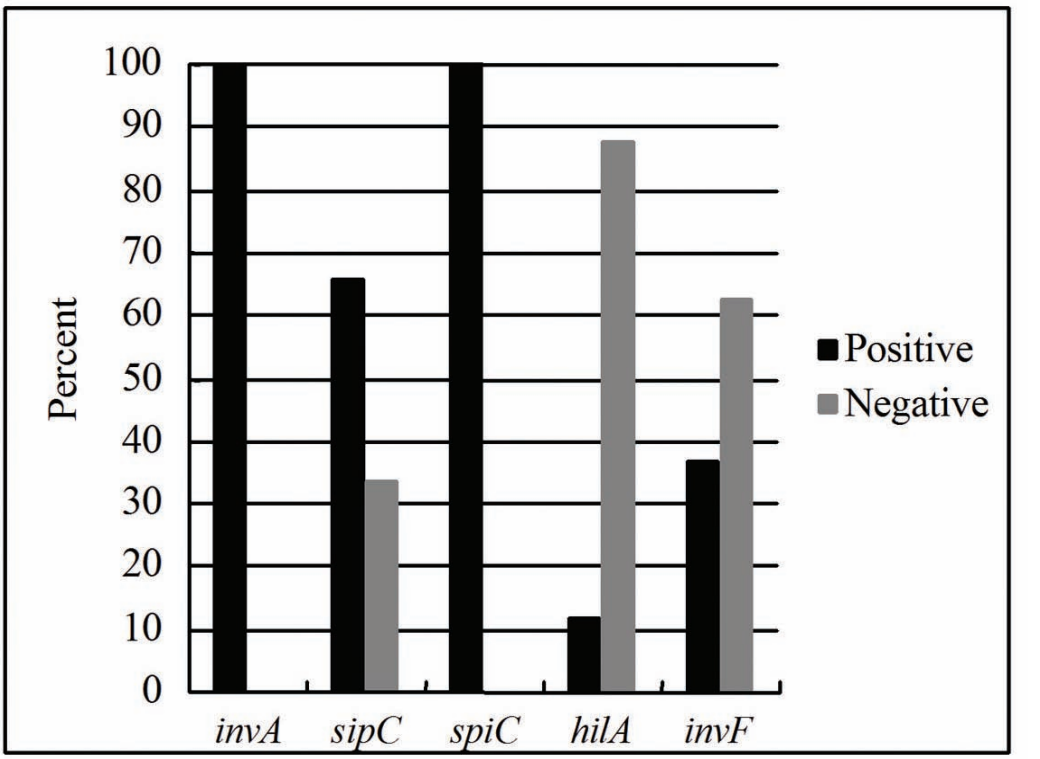


Figure 2. The presence of invA, *sipC*, *spiC*, *hilA*, and *invF* in the *Salmonella* isolates examined from eggs, chicken, and beef carcasses expressed as percent positive and negative.



pathogenicity island 2 effectors.[12,19] The *spiC* protein has also been previously shown to be a translocated effector that contributes to virulence.[19] In addition, *invF* and *hilA* expressed in response to environmental signals,[13] was low in poultry isolates indicating a variation between strains. The present study has confirmed that dot blot hybridization technique can be used to characterize *Salmonella* isolates as to weather they possess virulence determinants. These results can be utilized to yield a better understand-

Figure 3. The presence of various effector proteins and *invA* gene in 13 *Salmonella* isolates from beef carcasses expressed as percent positive and negative.

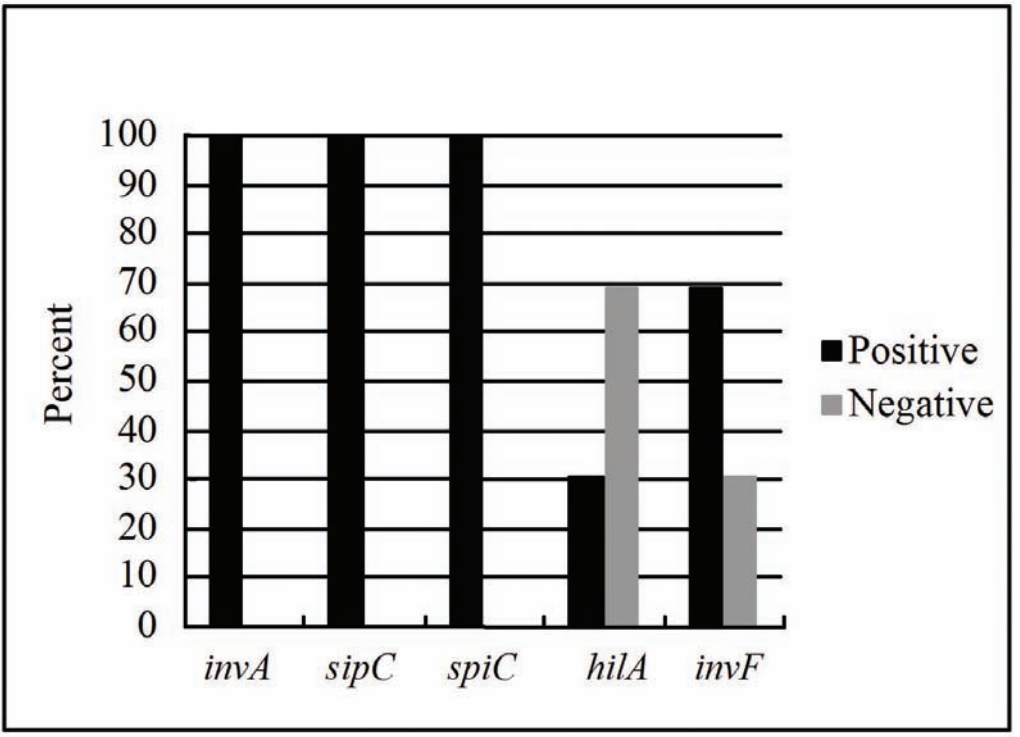
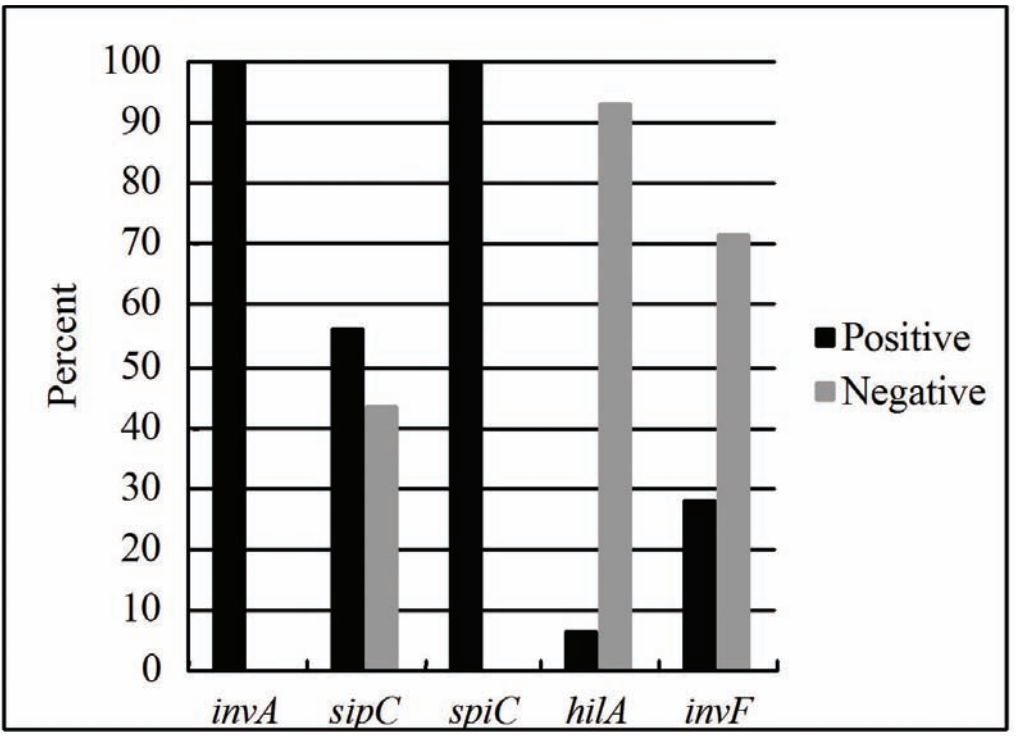


Figure 4. The presence of various effector proteins and *invA* gene in 46 *Salmonella* isolates from eggs and chicken carcasses expressed as percent positive and negative.



ing on the evolution of pathogenic serovars and the role of SPIs in the differences in pathogenesis and further epidemiological studies.

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