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Review

Feasibility of using dot blot hybridization to detect *Salmonella InvA*, *SpiC* and *SipC* directly from clinical specimens

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Pathogenesis of *Salmonella* depends upon a large number of factors controlled by an array of genes that synergise into actual virulence. The goal of this study was to detect *Salmonella invA*, *spiC* and *sipC* directly from clinical specimens, using the dot blot hybridization assay. We detected *invA* , *spiC* and *sipC* as a one combination from 4.5% (95% CI: 2.21 to 8.64) human feacal and 35.2% (95% CI: 26.4 to 45.0) poultry samples after enrichment. Furthermore the dot blot method had a higher sensitivity than routine culture, before and after enrichment. These results indicate that dot blot hybridization may be used to directly detect *Salmonella invA*, *spiC* and *sipC* in clinical samples.

Key words: *Salmonella*, dot-blot hybridization, *spiC*, *sipC*, *invA*, clinical-samples.

INTRODUCTION

Salmonellosis is one of the common causes of food borne diarrheal disease worldwide. Most of the infections are zoonotic in nature as they are usually transmitted from healthy carrier animals to humans. The main reservoir of zoonotic *Salmonella* is food animals, with sources of infections being animal-derived products (Kuhne and Lhafi, 2005), notably meat and eggs. In developing countries, contaminated vegetables, water and human-to- human transmission are also believed to contribute to a comparatively larger proportion of human cases. The incidence of human salmonellosis increased in most industrialized countries in the latter half of the 20th century due to the rapid spread of *Salmonella* in key areas of poultry meat and egg production units. Despite much research and many national and international attempts to implement control strategies, the incidence of human salmonellosis in most developing countries remains high (Thorns, 2000). In all the control programs involved, diagnosis of *Salmonella* remains of paramount importance. The common usual diagnostic methods of *Salmonella* are bacterial culture, polymerase chain reaction, ELISA and serological tests where vaccination is not done. New tools are always being sought to improve on the speed of diagnosis. The usual methods being used are at the same time being re-evaluated. In this study we tried to evaluate the direct detection of important elements responsible for the pathogenicity of *Salmonella* in clinical samples.

Salmonella enterica has been documented to have evolved a very sophisticated functional interface with its vertebrate hosts. This being the specialized organelle type III secretion system that directs the translocation of bacterial proteins into the host cell (Raffatellu et al., 2005; Wood et al., 1996). It is a two-component system that delivers a remarkable array of bacterial proteins capable

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of modulating a variety of cellular functions such as actin cytoskeleton dynamics, nuclear responses and endocytic trafficking secretion (Galan, 2001). These proteins are now presumed to be responsible for the pathogenicity and virulence of *Salmonella*. The proteins are encoded within the *Salmonella* pathogenicity islands (Schmidt and Hensel, 2004; Freeman et al., 2002) and may include *invA*, *sipA, sipB, sipC, sifA, hilA, hilC, hilD* and *invF* (Isogai et al., 2005; Amavisit et al., 2003; Bajaj et al., 1995; Galan and Curtiss, 1989). In this study, we tried the dot blot hybridization technique to detect directly the presence of *Salmonella spiC, sipC* and *invA* genes from clinical specimens of poultry and human origin. Furthermore, the samples were also subjected to the routine culture and isolation methods. Emphasis was mainly placed on the *invA* gene as it is thought to be linked with the virulence of *Salmonella* by triggering the invasion of bacteria into gastrointestinal epithelial cells (Darwin and Miller, 1999). It is therefore an important marker gene being targeted when detecting pathogenic *Salmonella* (Isogai et al., 2005; Boyd et al., 1997).

This study was carried out at the major diagnostic laboratories of Microbiology in Zambia, these being the University Teaching Hospital and the Faculty of Veterinary Medicine in Lusaka city, Zambia. The samples examined were from the *Salmonella* suspected clinical specimens submitted to the laboratories for bacteriological analysis. The samples included 200 diarrhea human feacal samples from the hospital and 108 specimens from the local poultry farms around Lusaka, Zambia. The samples from the chickens included dead in shell chicken embryos, liver, kidney and spleen, specifically submitted for *Salmonella* diagnosis.

The samples were subjected to the dot blot hybridization system for the detection of *Salmonella sipC*, *spiC* and *invA* genes. The DNA was extracted from the portion of the sample streaked directly on Xylose lysine deoxycholate agar (XLD; Oxoid, Hampshire, England). The sample was also inoculated into selenite enrichment broth. Furthermore a loopful of faecal or chicken sample was suspended in 100 µl of SDS buffer (1% SDS in 50 mM Tris-HCl, pH 8.0) and then boiled for 3 min, after which centrifugation at 9000 g for 5 min was done to extract the supernatant. In the case of chicken samples, they were first cut into small pieces using a sterilized fine scissors. After overnight incubation of feacal and chicken samples in selenite broth, 1 ml of broth was centrifuged at 1500 g for 10 min to harvest bacterial cells. Following centrifugation, the resultant pellet was treated with 100 µl of SDS buffer. The mixture was heated at 95°C for 3 min and then preceded to centrifugation as earlier mentioned. The dot blot hybridization assay was done using supernatant extracts as the antigen. These were dot spotted on the nitrocellulose membrane (Schleicher AND Schuell Bioscience, Dassel, Germany) by using a copy steel plate capable of transferring 25 µl of the supernatant extract per sample. The copy steel plate transfers 48 samples at a time. After transferring the antigen extracts, the membranes were allowed to dry at room temperature followed by blocking with 5% skim milk in PBS for 1 h at room temperature. The membranes were then washed 5 times in 30 min with PBS and then incubated for 1 h at room temperature with the first rabbit antibody against *sipC, spiC* and *invA* (1: 2,500 dilution) for each respective membrane in 0.5% skim milk. Following incubation, the membranes were washed and treated with goat anti-rabbit antibody (1: 5,000 dilution) for further 1 h at room temperature. After washing, the membranes were developed in 5 ml TMB (tetramethyl-benzidene) stabilized substrate for horseradish peroxidase (Promega Co., Madison, WI) until a positive reactive spot was visible in 5 min. To stop the reaction, the membranes were rinsed in distilled water and then air-dried. A known positive control sample of *Salmonella enteritidis* was included from a previous study (Hang'ombe et al., 1999) . The isolated *Salmonella* on culture was also grown in brain heart infusion broth and then subjected to the dot blot hybridization assay as earlier described.

For the isolation and identification of *Salmonella* standard conventional methods were used as described by Hang'ombe et al. (1999). Both samples from the hospital and local farms were cultured into selenite broth (Biotec, Suffolk, UK) for enrichment. Approximately a gram of the sample was placed in 10 ml of selenite broth and then incubated at 37°C for 18 h. The samples were also directly inoculated on the XLD agar plates without enrichment as mentioned previously. After overnight incubation *Salmonella* suspected colonies were confirmed by the polyvalent O grouping antiserum (Denka, Tokyo, Japan).

In this study the direct detection of *Salmonella* effectors before and after enrichment in selenite broth was evaluated. The basis of determining a positive reaction on the membranes is shown in Figure 1 by spots. The rate of detection in feacal samples was much lower compared to the poultry specimens. The results demonstrated the high prevalence of *Salmonella* in poultry samples being submitted to the laboratory. There was an improvement in the detection rate of *sipC*, *spiC* and *invA* after enrichment as shown in Table 1 and Table 2. This is as expected and demonstrated by the culture results as well. In addition the detection rate of *Salmonella* using the dot blot hybridization assay was higher than that observed under culture where 4.5% (95% CI: 2.21 to 8.64) and 38.9% (95% CI: 29.8 to 48.8) were culture positive in feacal and chicken samples after enrichment respectively. The dot blot technique was more sensitive than the routine culture method in both samples. This could be attributed to the targeted materials, such the presence of the effector proteins even after the death of *Salmonella* cells, could be observed in the detection of *invA* which was higher before and after enrichment. The high percentage of detecting *invA* in poultry is interesting, as this gene is

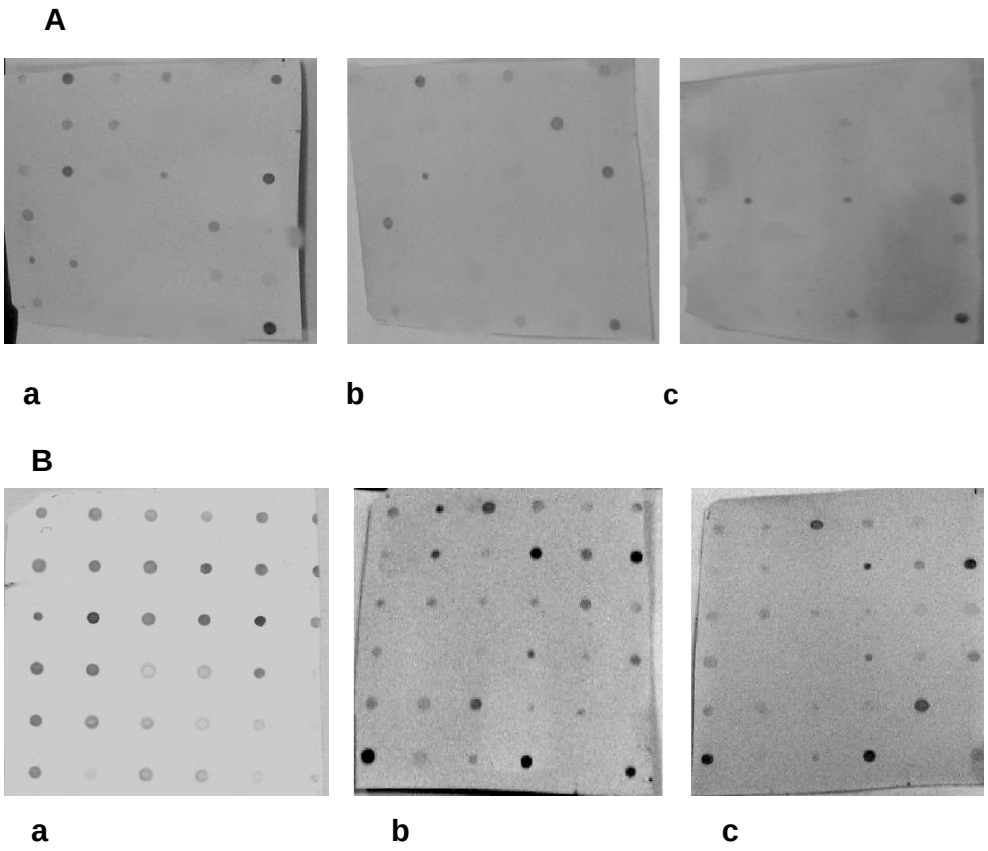

Figure 1. Dot blot hybridization for the visual detection of *invA*, *spiC* and *sipC* in feacal (A) and poultry (B) samples. Antigen extracts were prepared from samples and then dot spotted on the nitrocellulose membranes. The membranes were immunostained with specific antibodies against anti-*invA* (a), anti-*spiC* (b) and anti-*sipC* (c). Typical positive reactions are shown with deep staining spots.

Table 1. Total number of human samples reacting positive to *invA*, *sipC*, *spiC* and culture method.

***Salmonella* detection**[a]	**Human feacal samples (n = 200)**	
	No. positive on direct examination (%; 95% CI)	**No. positive after selenite enrichment (%; 95% CI)**
sipC	1 (0.5; 0.2-3.18)	16 (8.0; 4.79-12.9)
spiC	2 (1.0; 0.17-3.94)	14 (7.0; 4.03-11.7)
invA	5 (2.5; 0.92-6.06)	25(12.5; 8.40-18.1)
spiC, *invA*	0 (0)	11 (5.5; 2.92-9.89)
sipC, *invA*	0 (0)	9(4.5; 2.21-8.64)
sipC, *spiC*	0 (0)	10 (5; 2.56-9.23)
spiC, *sipC*, *invA*	0 (0)	9(4.5; 2.21-8.64)
Culture, *spiC*, *sipC*, *invA*,	0 (0)	7(3.5; 1.54-7.37)
Culture	1 (0.5; 0.20-3.18)	9(4.5; 2.21-8.64)

[a]The detection of *Salmonella* based on the effector proteins and routine culture method.

quite significant in propagating systemic *Salmonella* infections by enabling the bacteria survive and replicate within macrophages (Hansen and Hensel, 2001; Cirillo et al., 1998). The observation is true under these results, since poultry samples were obtained from systemically infected chickens. Furthermore, *Salmonella* in faecal specimens could not be detected as there were some inhibitors found in the gastro intestinal tract. The genes

Table 2. Poultry samples reacting positive to *invA*, *sipC*, *spiC* and culture method.

***Salmonella* detection**[a]	**Chicken samples (n = 108)**	
	No. positive on direct examination (%)	**No. positive after selenite enrichment**
sipC	23 (21.3; 14.2-30.4)	41 (38.0; 28.9-47.8)
spiC	21 (19.4; 12.7-28.4)	47 (43.5; 34.1-53.4)
invA	26 (24.1; 16.6-33.4)	52 (48.1; 38.5-57.9)
spiC, *invA*	16 (14.8; 8.96-23.2)	45 (41.7; 32.4-51.6)
sipC, *invA*	18 (16.7; 10.4-25.3)	39 (36.1; 27.3-46.0)
sipC, *spiC*	22 (20.4; 13.5-29.4)	40 (37.0; 28.1-46.9)
spiC, *sipC*, *invA*	20 (18.5; 11.9-27.4)	38 (35.2; 26.4-45.0)
Culture, *spiC*, s*ipC*, *invA*,	14 (13.0; 7.52-21.1)	34 (31.5;23.1-41.2)
Culture	14 (13.0; 7.52-21.1)	42 (38.9; 29.8-48.8)

[a]The detection of *Salmonella* based on the effector proteins and routine culture method.

understudy can be used as diagnostic markers, since they occur as clusters of chromosomal virulence genes found only within the genus *Salmonella* (Freeman et al., 2002). Comparing the dot blot hybridization detection results matched together as culture-*spiC-sipC-invA* and *spiC-sipC-invA* in poultry samples, a detection percentage of 31.5% (95% CI: 23.1 to 41.2) and 35.2% (95% CI: 26.4 to 45.0), respectively was observed. The results indicate the effectiveness of the dot blot hybridization in detecting pathogenic *Salmonella*. It is therefore important to note that the dot blot hybridization system can detect *Salmonella* cells that may not propagate even after enrichment. On the other hand, we could clearly see that on culture some of the *Salmonella* isolated could not be detected on the dot blot assay. This could be attributed to *Salmonella* not being pathogenic in nature or not carrying any of the pathogenic factors under investigation. All the culture positive samples were found to be *spiC, sipC* and *invA* positive except for two and eight samples from the human and chickens respectively.

In general, the dot blot hybridization may be used to determine the accuracy of isolating pathogenic *Salmonella*. The detection of *Salmonella* effector proteins directly from clinical specimens especially poultry clearly shows that an easy and quick technique can be used to assess the bio-quality of poultry products.

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