Comparative examination and validation of *Salmonella* immuno dot-blot strip in poultry samples
(Ujian perbandingan dan pengesahan jalur pemendapan imuno-titik *Salmonella* pada sampel ayam)

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Abstract
Effective and rapid monitoring of *Salmonella* in the poultry production chain is necessary to assure safe food products for consumers. Previously, an immuno dot-blot test strip based on polyclonal antibody against *Salmonella typhimurium* was successfully developed. However, validation is needed to ensure that their performance is equal to established methods. Thus, a comparative study of the diagnostic immuno dot-blot procedure with the standard culture-based method using *Salmonella* selective chromogenic agar and other commercially available kits were performed. Samples of refrigerated chicken cuts were spiked with *S. typhimurium* before being tested for specificity, sensitivity, and cross-reactivity of the test strip. The results showed that the immune dot-blot strip was comparable with *Salmonella* Chromogenic agar and commercial kit. To determine the specificity of the strip, cross-reactivity between four species of the Enterobacteriaceae family and *Salmonella* as an indicator was carried out. This strip exhibits high selectivity against *V. cholerae*, *L. monocytogenes* and *Shigella*, although cross-reaction was observed with *E. coli*. The results obtained revealed that immuno dot-blot strip gave comparable results with existing *Salmonella* detection methods in terms of time and accuracy. Thus, these results proved this strip is useful as a rapid diagnostic test for the direct detection of *Salmonella* in food.

Keywords: *Salmonella*, validation, rapid detection, immune-dot blot, poultry

Introduction
Food-borne diseases such as salmonellosis are recognised as one of the most serious public health concerns today (Tirado and Schmidt 2001). The World Health Organization (WHO) reported that salmonellosis caused by *Salmonella* sp. is the most frequently reported food-borne diseases worldwide (Schlundt 2002). A variety of food products, especially poultry and other type of meat products are the most important sources of human salmonellosis infection (Chittick et al. 2006). Although food is an essential part of a human daily life, food safety remains a concern as foods have been known to be the vehicles for transmitting infectious diseases. In addition to the problem of food-borne illness, losses due to microbial spoilage and contamination in foods usually have a significant economical impact on the country producing it (Tothill and Magan 2003).

The conventional culture method used today for detection of *Salmonella* in food is laborious and takes 3 – 7 days to complete. In order to avoid the sale of contaminated products, expensive inventories are held at the production site while samples are tested for microbial contamination, which often takes more than 3 days. Since food products...
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have short shelf life, they are released before microbial results are available (Tothill and Magan 2003). Therefore, the ability to rapidly detect and identify this pathogen is extremely important to maintain public health safety and security.

Various methods have been developed and are used for the detection of *Salmonella* sp. Conventional culture methods involve blending of the food product in a pre-enrichment media to increase the population of the target organism, followed by plating onto selective or differential agar plates to isolate pure cultures. These are then examined by phenotypic analysis or metabolic markers. A major drawback is that these methods are labour-intensive and also take 2 – 3 days for the results to be known and up to 7 – 10 days for confirmation (Tothill 2006). Methods based on nucleic acid probes and polymerase chain reactions (PCR) have also been used although the time frame of the analysis is still several hours and requires trained personnel to conduct the assays (Mozola 2006). Recently, an immuno dot-blot test strip based on polyclonal antibody against *S. typhimurium* was developed for the detection of *Salmonella*. The immuno dot-blot is a rapid and sensitive immuno-enzymatic detection method for specific peptides immobilized on nitrocellulose polyvinylidene fluoride membrane. It is considered to be one of the most promising techniques to meet this demand, and several immuno dot-blot based detection methods for *Salmonella* in food and feed have been developed (Hang’ombe et al. 2008).

Validation is an important step in the process of standardising a method because it provides evidence that the new method gives results at least as good and in agreement with the currently used reference method, as well as proving confirmation of the reproducibility and specificity when used by other laboratories (Malorny et al. 2003).

These data are needed to gain acceptance among authorities and end users of a method and to speed up the implementation of immuno dot-blot detection systems in diagnostic laboratories. The present study attempted to perform a comparative study of a diagnostic immuno dot-blot procedure and the currently used *Salmonella* Selective Chromogenic Agar reference method for the detection of *Salmonella* in poultry samples.

**Materials and methods**

**Materials**

Refrigerated chicken cuts (drumsticks, wings and ribs) were purchased from local supermarkets. The samples were analysed immediately upon arrival at the lab. A total of 50 chicken parts were assessed.

**Growth in enrichment media**

Samples were cut into small pieces, weighed and autoclaved for 15 min at 121 °C. Each sample weighed 25 g was homogenised with 225 ml of *Salmonella* media (Strategic Diagnostics Inc. USA) in a stomacher 400 (Seward Medicals, Worthing, UK) for 30 s and incubated at 42 °C for 20 h. After incubation, 1 ml of enriched samples were transferred into tetrathionate broth (TTBB) (OXOID, UK) and incubated at 42 °C for 20 h.

**Organisms**

*Salmonella typhimurium*, *E. coli*, *V. cholera*, *L. monocytogenes* and *Shigella* were obtained from the Biotechnology and Nanotechnology Research Centre, MARDI.

**Sensitivity tests**

Samples were divided into two sets: A (negative samples) and B (positive samples). Set B were spiked with *S. typhimurium* while samples in Set A were left untreated. The spiking process was performed by adding one loop of bacteria from the *S. typhimurium* pure culture into the *Salmonella* media containing the chicken
samples. The density of one loop of *S. typhimurium* was determined at $10^9$ CFU/ml. After the incubation period ended, the enriched aliquots were then tested for the presence of *Salmonella* using *Salmonella* immuno dot-blot strip and validated using *Salmonella* Selective Agar (OXOID, UK) and commercially available *Salmonella* Detection Kit.

**Cross-reactivity tests**
Cross reaction tests with other Enterobacteria was conducted using *E. coli*, *V. cholera*, *Shigella* and *L. monocytogenes*. Positive control comprises of samples spiked with *S. typhimurium* and negative control (free from *S. typhimurium*). Samples were spiked during the enrichment step as described above. The samples were then validated with commercially available *Salmonella* detection test kit and the *Salmonella* Selective Agar.

**Salmonella detection methods**
The presence of *Salmonella* was detected using *Salmonella* immuno dot-blot strip. *Salmonella* Selective Agar and commercially available *Salmonella* detection kits were also used to corroborate the results.

**Salmonella immuno dot-blot strip**
This strip is a simplified version of ELISA test. The test was performed by dropping 5 µl of TTBB onto the strip’s membrane and left to dry. The membrane was then blocked with 0.5% dry milk for 1 h at room temperature. This was followed by incubation with the primary antibody for 30 min at room temperature. Following the incubation, the membrane was washed with phosphate buffered saline with tween (PBST). The membrane was further incubated with alkaline phosphatase (Sigma, USA) for 30 min at room temperature. Next, the membrane was washed three times with PBST followed by a single rinse with phosphate buffered saline (PBS). The final step involves incubation with BCIP (Thermo Scientific, USA) until BCIP (Thermo Scientific, USA) until colour development was observed. Positive (+) results were defined by the development of dark blue colour in the sample wells.

**Commercially available Salmonella detection kit**
The tests were carried out with GLISA Singlepath® *Salmonella* (Merck, Germany), as per manufacturer’s instructions.

**Salmonella Selective Agar**
At the end of selective enrichment, 100 µl of the broths were streaked onto *Salmonella* Selective Agar (OXOID, UK), and incubated at 37 °C for 48 h to isolate the suspected colonies. Positive (+) results indicated the growth of *Salmonella* colony was detected.

**Results and discussion**

**Sensitivity tests**
Traditionally, the detection of *Salmonella* in poultry by culture method has been relying on multiple enrichment steps. This approach however was found to be laborious and time consuming. The chief advantage of immuno dot-blot strip over the culture method is that the former method is simple and can handle large number of samples simultaneously. Leung et al. (2001) reported that commercial detection kits for food pathogen demonstrate varying accuracies which may due to different types of bacteria species isolated from different geographic regions used for the coating antigen preparations. Prior to this an immuno dot-blot test strip based on polyclonal antibody against *S. typhimurium* was successfully developed in our lab. This strip was able to give results qualitatively within 30 min and no special equipments were required to analyse the results.
According to Rijpens et al. (1999) the selection of a diagnostic test depends not only on test features, such as sensitivity and speed, but also on extrinsic factors, like food type, stress on organism and interpretation of results.

Sensitivity measures the proportion of positives that are correctly identified by a method, in this case positive test portions for *Salmonella*. The negative result for *Salmonella* Agar and *Salmonella* strip is presented in Figure 1(a) and (b) while the basis of determining a positive reaction is shown in Figure 2. A positive reaction in Figure 2(a) is defined by the growth of purple *Salmonella* colonies on *Salmonella* agar while Figure 2(b) by purplish-blue spots on *Salmonella* strip distinguishable from the colourless area of negative control. Sensitivity results for the *Salmonella* strip, the conventional method and the commercial kit are illustrated in Figure 3. From the results obtained, 50% of the samples tested in Set A were found to be reactive by the *Salmonella* strip (Figure 3). On the contrary, bacterial growth was not observed in all samples cultured on *Salmonella* agar and the commercial kit. Nevertheless, in Set B, *Salmonella* was recognised by the *Salmonella* strip and the commercial kit (Figure 3). Then again, we could clearly see that the presence of *Salmonella* could not be detected on some cultures as compared to the dot blot assay and commercial kit. Discrepancies among the sensitivity of conventional agar method may be due to improper sampling which may drop the level of detection below the threshold of detection. Conventional culture method could detect only living cells while the dot-blot strip detected any form of *Salmonella* antigen. Moreover, positive results by the culture method require that two conditions are met. First, a sufficient number of salmonellae must be present in the selective enrichment step to assure that one or more *Salmonella* cells in aliquots streaked onto the plate. Second, the relative proportion of salmonellae to other organisms that are capable of growing in/on the selective/differential media must be such that at least one isolated colony of *Salmonella* can be obtained. Thus, obtaining a positive result by the culture method is based primarily on the chance of selectivity, while a positive result in the dot-blot depends more on actual numbers of salmonellae or the amount of their antigen presented. The present study indicated that both commercial kit and immuno dot-blot strip methods are comparable in efficiency for most of the samples tested. The immuno dot-blot strip included in the study emerged as an alternative to culture method and the commercial kit for the detection of *Salmonella* in poultry products. Additionally, the immuno dot-blot strip was more rapid than the culture method since it could handle more samples at a time. It is also more economical than the commercial kit because it was made locally.
Figure 1. Negative results for (a) Conventional method (Salmonella Agar) (b) Salmonella strip

Figure 2. Positive results for (a) Conventional method (Salmonella Agar) (b) Salmonella strip
Cross-reactivity tests

Salmonella typhimurium is one of the most commonly isolated causative agents of acute gastroenteritis in humans and farm animals. Its precise diagnosis is important for epidemiological investigation. Cross reaction is defined as the interaction of an antigen with an antibody formed against a different antigen with which the first antigen shares identical or closely related antigenic determinants. Reports by Peterfi et al. (2007) and Magliulo et al. (2007) have described cross reactions between E. coli and Salmonella antigens. Cross reactions between E. coli and Shigella were also reported by Bin Liu et al. (2008).

The results were reported based on the experiment conducted to test the efficiency of the developed immuno dot-blot method in detecting other types of bacteria other than Salmonella in food. Bacteria chosen were namely E. coli, V. cholera, Shigella and L. monocytogenes as they have similar characteristics to the genus Salmonella.

In this experiment, chicken meat samples were spiked with either E. coli, V. cholera, Shigella or L. monocytogenes. It also comprises of positive control (spiked with S. typhimurium) and negative control (samples were autoclaved to keep it free from S. typhimurium). All of the samples were then subjected to 2 steps of enrichment. Samples were then examined for cross reactions with Salmonella using standard culture method and counter-checked using Salmonella immuno-dot blot strip and commercial kits.

The results are shown in Figure 4. It was observed that for positive and negative controls, the three methods correlated with each other. Concurrently, cross reactions between Salmonella and E. coli were detected through the positive results indicated by the Salmonella strip and Salmonella agar. This finding stressed the well-known close relationship between E. coli and Salmonella. However, isolates of V. cholera did not exhibit a positive reaction when tested using the Salmonella strip.

*Results are based on 50 chicken parts tested.

Figure 3. Comparison of methods used to detect Salmonella in Set A (negative sample, free from Salmonella) and Set B (positive sample, spiked with Salmonella)
This finding is comparable to a study by Tuteja et al. (2007) who reported that no cross reaction was observed when *S. typhimurium* was tested with rabbit anti-*V. cholera* polyclonal antibody. The same trend was also demonstrated in samples spiked with *Shigella* and *L. monocytogenes* where the three methods employed.

It was identified that *L. monocytogenes* and *Shigella* were undetected by *Salmonella* strip and commercial kit. On the contrary, both members of the Enterobacteriaceae family gave positive results when tested using conventional method. The dissimilarities of the data between culture method and the strip might be due to the usage of *Salmonella* agar as an identification medium. The agar is commonly used to isolate *Salmonella* and *Shigella*. Although, the medium contains bile salts to inhibit the growth of Gram-positive organisms, the growth of *L. monocytogenes* could be due to contamination during the experimental process.

**Conclusion**

The strip validated in this study enabled the detection of *Salmonella* in less than 24 h, compared to at least 2 days using the *Salmonella* Selective agar. The simplicity and the speed of the strip make it suitable for routine analysis for large number of samples and the implementation of the method in industry will help improve safety in the food production chain.

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**Figure 4. Cross reactivity results**

<table>
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References


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