ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

International Journal of Poultry Science 9 (9): 831-835, 2010
ISSN 1682-8356

Rapid Detection of *Salmonella* in Chicken Meat Using Immunomagnetic Separation, CHROMagar, ELISA and Real-time Polymerase Chain Reaction (RT-PCR)

Ensaf G. Taha, A. Mohamed, K.K. Srivastava and P.G. Reddy
Department of Pathobiology, College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University, Tuskegee, AL, USA

Abstract: The main objective of this study was to standardize and compare rapid methods for the detection of *Salmonella* in meat samples using Immuno-Magnetic Separation (IMS) followed by culturing in CHROMagar Plus media, Enzyme-Linked Immunosorbent Assay (ELISA) and Real-Time Polymerase Chain Reaction (RT-PCR). Ten-fold dilutions of bacterial suspension (*S. typhymurium*, ATCC13311) were prepared from the original concentration of 1.6 x 10^6cfu/ml. Chicken wing samples of 25 g each negative for *Salmonella* were spiked with six different concentrations of bacteria ranging from 10^6 to 10^1. These samples were incubated in buffered peptone water for 4 h as pre-enrichment step and were tested repeatedly. The IMS technique involved the use of paramagnetic polystyrene microscopic beads coated with purified antibodies against *Salmonella*. The CHROMagar Plus media containing chromomeric substrate facilitated detection of *Salmonella* species from other flora. The Assurance EIA *Salmonella* Kit with polyclonal antibodies directed against *Salmonella* facilitated easy and rapid detection. In the RT-PCR primers targeting *invA* gene was used which amplified a 378 bp fragment. Comparing to conventional culture method (4 days), CHROMagar plate culture following IMS showed light mauve to mauve-colored colonies of *Salmonella* in 23 h with high sensitivity (99%) at 1.6 cfu/ml. IMS-ELISA combination also showed high sensitivity (75%) at 1.6 x 10^3 cfu/ml in 8 h and minimized cross-reactivity with many *Enterobacteraceae*. The combination of IMS with RT-PCR took less than 7 h and was even more sensitive (100%) at 1.6 cfu/ml. Sensitivities of IMS-RT-PCR and IMS-CHROMagar were higher compared to IMS-ELISA. IMS-CHROMagar was easier to perform and detects only living *Salmonella*. These methods will be highly suitable for routine detection and may significantly assist the processing industry in avoiding costly recalls and the timely investigation of outbreaks.

Key words: *Salmonella*, rapid detection, immunomagnetic separation, ELISA, RT-PCR, CHROMagar

INTRODUCTION

Outbreaks of foodborne salmonellosis in recent years have raised serious concern regarding rapid diagnosis of the pathogen with high specificity and sensitivity. The time consumed in the conventional methods has been the driving force behind development of rapid tests. The incubation periods needed for the pre-enrichment step, culture media, biochemical and serological tests used in the conventional methods taking 24 h for each step makes the total of 4 days to confirm the presence of *salmonella*. It is also important to distinguish *Salmonella* colonies from other *Enterobacteraceae*. because it may result in false positives. MacConkey agar, Salmonella-Shigella agar (SS) and Rambach are the selective media used for isolation of Gram-negative enteric bacteria. The chromogenic characteristic of (RAM) involves fermentation of D-glucuronate and a β-galactosidase indicator. It demonstrated high levels of sensitivity and specificity and reduced the time to final identification of *Salmonella* (Maddocks *et al*., 2002) Serological tests such as Enzyme-Linked Immunosorbent Assay (ELISA) are used in a number of countries in which antibodies are used which target specific *Salmonella* antigens, without the cells being viable. Immunomagnetic Separation (IMS) technique using anti-Salmonella polystyrene beads appears to solve the problems of samples containing low resident flora or concentrating the target bacteria from a large volume to a volume suitable for detection by culture, ELISA or molecular methods. Agarose gel electrophoresis accompanied by ethidium bromide staining was used in the conventional PCR showed some risk of carry-over contamination in the laboratory, labor and time intensive. Real-time PCR (RT-PCR) using non specific fluorescent double-stranded DNA-binding dyes such as Sybr Green allows highly sensitive detection in a few hours with minimal handling of the samples. *InvA* gene contains sequences unique to *Salmonella* and this gene is a suitable PCR target, with potential diagnostic applications (Galan *et al*., 1992).

The objective of this study was to standardize and compare rapid, specific and sensitive methods based on immunomagnetic separation technique followed by chromogenic selective media, ELISA and RT-PCR.

Corresponding Author: P.G. Reddy, Department of Pathobiology, College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University, Tuskegee, AL, USA

These methods are compared with the standard culture method in terms of reducing the detection time and increasing the sensitivity. These methods would be useful in decreasing the duration of outbreaks, lowering the medical costs and avoiding costly recalls.

MATERIALS AND METHODS

Salmonella typhimurium (ATCC 13311) was used to prepare ten-fold dilutions of *Salmonella* suspension ranging from 10^6 to 10^1 from the original concentration of 1.6 x 10^6 cfu/ml. These 6 dilutions were used to spike *Salmonella*-negative chicken wing samples collected from local retail stores and were subjected to conventional culture as well as Immuno-Magnetic Separation (IMS) using anti-*Salmonella* polystyrene beads (DYNAL Biotech, Carlsbad, CA). IMS samples were then tested by CHROMagar Plus (CAS) (Becton Dickinson, Sparks, MD), Assurance EIA *Salmonella* ELISA (BioControl System, Inc, Bellevue, WA) and RT-PCR (One Step PCR Screening Kit; Takara Bio, Inc, Foster City, CA) using Sybr Green master mix (Applied Biosystem, Inc, Foster City, CA).

Sample preparation: Chicken wing samples of 25 g each, negative for *Salmonella* by culture were spiked with the six different concentrations (10^6, 10^5, 10^4, 10^3, 10^2, 10^1) of *S. typhymurium* 1.6x10^{1-6} cfu/ml. These samples were incubated in Buffered Peptone Water (BPW) for 24 h as pre-enrichment step for the standard culture method and samples with the same concentrations were incubated in BPW for 4 h as pre-enrichment step before subjected to IMS and tested by CHROMagar, ELISA and RT-PCR.

Conventional culture: The protocol of the United States Department of Agriculture/Food safety and Inspection Services (USDA/FSIS) MLG 4.03 (Isolation And Identification of *Salmonella* From Meat, Poultry And Egg Products) was used. MacConkey agar and *Salmonella-Shigella* agar (SS) were used as selective media to identify *Salmonella*. Briefly, the method involved 4 steps of approximately 24 h each. These were: 1) pre-enrichment in BPW. 2) Selective-enrichment. 3) Bacterial culture on agar and 4) - Biochemical and serological tests.

Immunomagnetic separation (IMS): The procedure for IMS was essentially as described by the manufacturer (DYNAL Biotech, Cat No. 710.02). Briefly, anti-*Salmonella* paramagnetic polystyrene beads (20 µl volume) were added to the spiked chicken wing samples (One ml from the filtered sample) / as the tubes were incubated at room temperature for 10 min. The antibodies coated onto the beads bonded to *Salmonella* antigen forming the bead-bacteria complex. Describe in one line collection of beads. Four groups of samples of all dilutions were used in ELISA, RT-PCR and CHROMagar. These were; 1) meat samples without bacteria and w/o IMS, 2) bacterial suspension w/IMS & w/o meat sample, 3) spiked meat sample w/IMS and 4) spiked food sample w/o IMS.

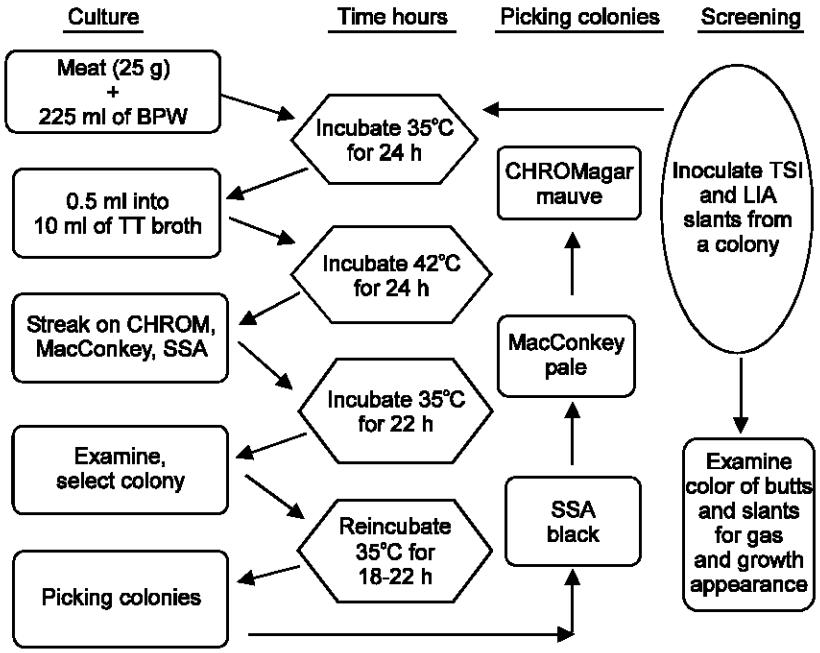

Fig. 1: Flow chart showing the conventional method (USDA) procedures

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bl K 0.19	Bl K 0.17	6 0.35	6 0.32	6 0.34	6 0.40	6 0.33	6 0.31	6 0.23	6 0.18	--- 0.09	--- 0.08
B	std 2.35	std 2.58	5 0.49	5 0.48	5 0.49	5 0.53	5 0.35	5	5 0.29	5 0.27	--- 0.08	--- 0.07
C	std 2.58	std 2.58	4 0.55	4 0.53	4 0.63	4 0.69	4 0.38	4 0.37	4 0.32	4 0.36	--- 0.09	--- 0.08
D	std 2.58	std 2.58	3 0.67	3 0.65	3 0.81	3 0.80	3 0.39	3 0.42	3 0.37	3 0.39	--- 0.08	--- 0.09
E	std 2.58	std 2.58	2 1.49	2 1.33	2 1.87	2 1.93	2 0.49	2 0.46	2 0.48	2 0.43	--- 0.08	--- 0.09
F	std 2.58	std 2.58	1 1.99	1 1.89	1 2.43	1 2.51	1 0.59	1 0.67	1 0.98	1 0.91	--- 0.09	--- 0.08

ELISA Plate showing positive result. (0.8 OD) considered +ve

Fig. 2: *Salmonella* ELISA plate schematic showing positive and sample readings from standards and samples

CHROMagar culture: CHROMagar *Salmonella* (CAS) was supplied as dehydrated culture medium, which was prepared according to the manufacturer's instructions (Becton, Dickinson Company). One loopful of inoculum of IMS samples was streaked onto CAS plates. The plates were incubated at 37°C for 23 h and were examined for the presence of *Salmonella* colonies.

Enzyme-Linked Immunosorbent Assay (ELISA): Assurance *Salmonella* EIA Kit was used according to the manufacturer's protocol (BioControl System, Inc, Cat No. 2000-01, 5000-01). The monoclonal antibody present on the plate wells selectively binds to *Salmonella* antigen in the IMS samples and then coated with assure-link antibody forming the sandwich ELISA which was detected by antibody-enzyme conjugate. The assay recognizes the bacterial antigen without the cells being viable. Microwell plate reader (Bio-Tec Instruments Inc, Winoosk, Vermont, USA) with 405 nm filter was used (absorbance values greater than or equal to the cut off value of 0.8 indicated by yellow color on the ELISA plate).

Real Time-Polymerase Chain Reaction (RT-PCR): The IMS beads were re-suspended in 100 µl of PBS-Tween and the genomic DNA was extracted by mixing 4 µl of the suspension with 196 µl of nuclease free water then heated at 95°C for 5 min using a thermal cycler and centrifuged at 12,000 x g for 10 min at 4°C. DNA was also extracted from positive control *S. typhimurium* (ATCC 13311) and from the spiked-meat samples, using 1ml from all dilutions (10^{6}-10^{1}). RT-PCR was performed using oligonucleotide primers SIN-1 and SIN-2 targeting *invA* gene of *Salmonella* species with concentration of 19 µm/53 µl, designed by Takara Bio Inc (Madison City, Wisconsin), These primers amplify a 378 bp product and react well with 174 strains of *Salmonella*. The primers were optimized according to the Applied Biosystems Sybr Green master mix based on the published validated real-time PCR for *Salmonella* detection (Nam *et al*., 2005). Reactions were run on a Smart Cycler II instrument (Cepheid, Sunnyvale, California). Melting curve was obtained after the last amplification cycle. C_T was determined when the level of fluorescence exceeded the threshold line. The point at which the growth curve intercepts the threshold line determines the value of the C_T, the lower the C_T the higher the concentration of the template DNA.

RESULTS

IMS method facilitated the concentration of the bacteria from spiked chicken samples as low as 1.6 cfu/ml in 1 h following the 4 h pre-enrichment in BPW. Using IMS concentrate, CHROMagar showed light mauve to mauve-colored colonies in 18 h with samples at 1.6 cfu/ml of bacterial suspension and at 16 cfu/ml from spiked meat sample with lower detection limit probably due to the composition of the food matrix. Using IMS concentrate, ELISA provided results in 3 h with (absorbance values greater than or equal to the cut off value of 0.8) at 1.6 x 10^{4} cfu/ml from spiked meat sample and at 1.6 x 10^{3} cfu/ml from bacteria suspension. Using IMS concentrate in RT-PCR the indicator Sybr Green I dye melting curve showed an over lapping of the

Time required for completion of each procedure		
4 days		
Standard method	Highest dilution detected in case of the bacterial suspension was 1.6×10^3 cfu/ml on *Salmonella-Shigella* agar and 1.6×10^4 cfu/ml in spiked food samples on MacConkey agar	Pre-enrichment (24 h) Selective-enrichment (24 h) Bacterial culture on agar (24 h) Biochemical and serological (24 h)
23 h		
IMS +CHROMagar	Highest dilution detected with pure bacterial suspension was 1.6 cfu/ml and with spiked meat samples was 16 cfu/ml	Pre-enrichment (4 h) IMS (1 h) Plating + incubation (18 h)
8 h		
IMS + ELISA	Highest dilution detected with pure bacterial suspension was 1.6×10^3 cfu/ml and with spiked meat samples was 1.6×10^4 cfu/ml	Pre-enrichment (4 h) IMS (1 h) ELISA (3 h)
7 h		
IMS + RT-PCR	Highest dilution detected with pure bacterial suspension was 1.6 cfu/ml and with spiked meat samples was 16 cfu/ml	Pre-enrichment (4 h) IMS (1 h) RT-PCR (2 h)

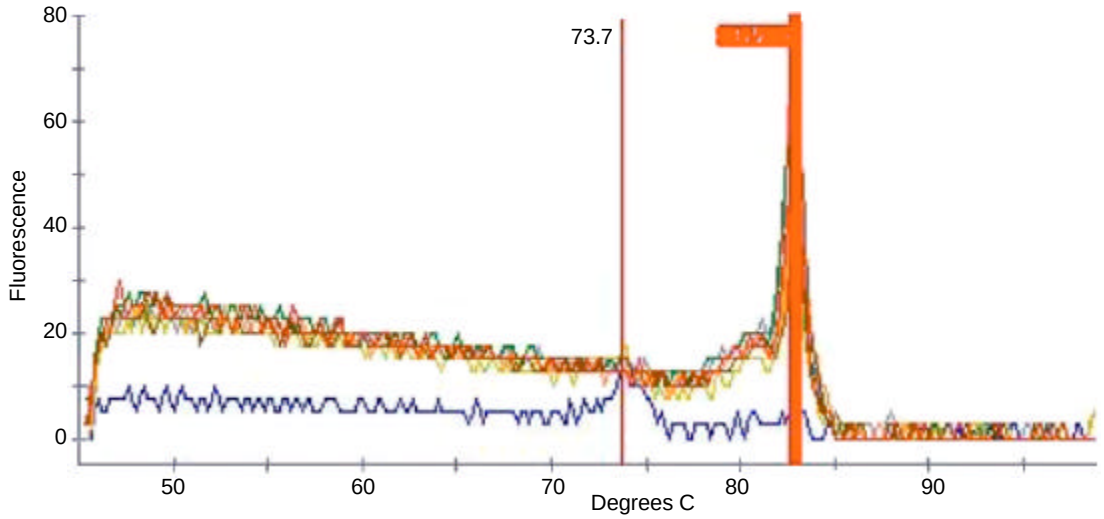

Fig. 3: RT-PCR melting curve obtained showing positive *S. typhimurium* melting curve temperature around 83°C which specific for the target

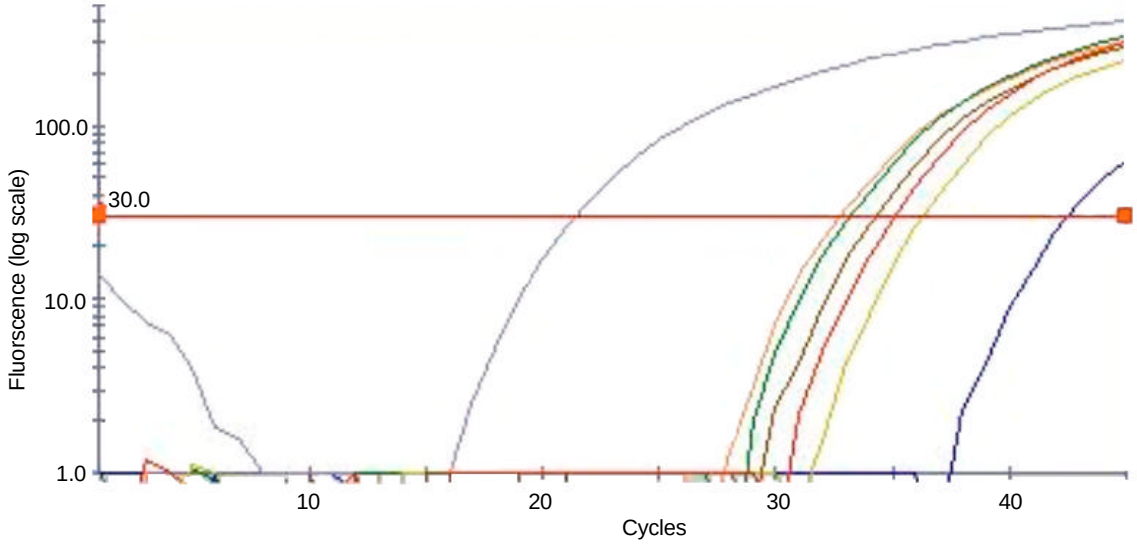

Fig. 4: Growth curve and amplification targeting invA gene. The growth curve represents fluorescence dye, which is directly proportional to concentration of the template DNA

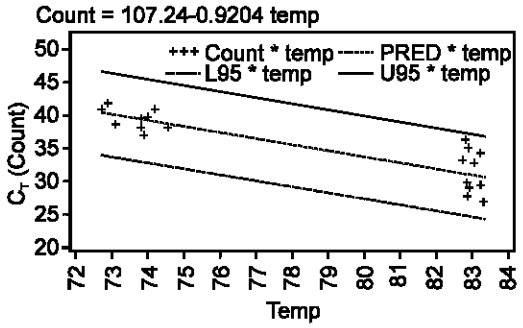

Fig. 5: Linear Regression Model showing high resolution of reading positive and negative samples. The correlation between dependent variable (C_T) or fluorescence dye and independent variable (melt-temperature) significantly showing that fluorescence dye is directly proportional to concentration of the template DNA

amplified products over the same temperature meaning the PCR assay amplified a distinct and specific product. Melting peak temperature of PCR product with the SIN 1 and 2 primers was 83°C. IMS followed by RT-PCR was able to detect (100%) of the positive samples from bacterial suspension as low as 1.6 cfu/ml and at 16 cfu/ml from the spiked food sample.

DISCUSSION

To our knowledge this may be one of the first studies that compared three different rapid methods following IMS on same meat samples. Reports using even a single method are scanty. In a study made by Kudo *et al.* (2001) the combination of IMS-CHROMagar method to detect *Salmonella* in eggs was successful except that the time consumed was 3 days to confirm the presence of *Salmonella*. In this study the same technique was used after 4 h pre-enrichment, the time consumed was 28 h to confirm the presence of *Salmonella*. Duncanson *et al.* (2003) used the combination of IMS-ELISA to detect *Salmonella* in food during an outbreak using environmental swab and feces samples. Sixty-seven percent of the samples were positive after 24 h pre-enrichment and 94% were positive following selective enrichment for additional 24 h. A similar combination procedure was used in this study with only 4 h pre-enrichment and we were able to obtain results with a sensitivity of (75%) at 1.6 x 10^3 cfu/ml in about 8 h. Mercanola and Griffins (2005) combined IMS-RT-PCR, targeting invA gene in different food products after 10 h of selective enrichment. *Salmonella* was detected within 13 h at 1 cfu/ml and at melting temperature of 87°C. The RT-PCR results obtained in this study showed a detection sensitivity of 100% at 1.6 cfu/ml when the samples were first subjected to IMS. The RT-PCR on spiked samples without IMS showed successful detection at 1.6 x 10^6 cfu/ml. Importantly, in this study only a 4 hour pre-enrichment step was used and less than 7 h was spent in all the steps to confirm the presence of *Salmonella*. The combination of IMS-CHROMagar method to detect *Salmonella* took 23 h to confirm the presence of *Salmonella* with a high level of sensitivity.

The combined methods of IMS-RT-PCR as well as IMS-ELISA described in this study are rapid methods suitable for the routine analysis of food samples. While IMS- ELISA was easy to perform, sensitivity of IMS-RT-PCR was much higher. Although IMS-CHROMagar takes more time than IMS-ELISA and IMS-RT-PCR, it reduced the time needed in the conventional culture method for confirmatory biochemical and serological tests by up to three days. The sensitivity of detection was much higher compared to IMS + ELISA. The major advantages with the IMS-CHROMagar method are that it only detects living *Salmonella* and is easier to perform.

REFERENCES

Duncanson, P.D., R.A. Wareing and O. Jones, 2003. Application of an automated immunomagnetic separation-enzyme immunoassay for the detection of *Salmonella* spp. During an outbreak associated with a retail premises. Appl. Microbiol., 37: 144-148. www.3.interscience.wiley.com/journal.

Galan, J.E., C. Ginocchio and P. Costeas, 1992. Molecular and Functional Characterization of the *Salmonella* Invasion Gene *invA*: Homology of *InvA* to Members of a New Protein Family. J. Microbiol., 174: 4338-4349.

Kudo, Y.H., S. Kumagai, T. Masuda, K. Goto and K. Ohtsuka, 2001. Detection of *Salmonella enteritidis* in shell and liquid eggs using enrichment and plating. Int. J. Food Microbiol., 64: 395-399.

Maddocks, S., T. Olma and S. Chen, 2002. Comparison of CHROMagar *Salmonella* Medium and Xylose-Lysine-Desoxycholate and *Salmonella-Shigella* Agar for Isolation of *Salmonella* Strains from Stool Samples. J. Clin. Microbiol., 40: 2999-3003.

Mercanola, B. and M.W. Griffins, 2005. Combination of immunomagnetic separation with real-time PCR for rapid detection of *Salmonella* in milk, ground beef and alfalfa sprouts. J. Food Prot., 68: 557-561. www.sproutnet.com/Research.

Nam, H., V. Srinivasan, B.E. Gillespie, S.E. Murinda and S.P. Oliver, 2005. Application of SYBR green real-time PCR assay for specific detection of *Salmonella* spp. In dairy farm environmental samples. Int. J. Food Microbiol., 102: 161-171. http://www.sciencedirect.com/science.

USDA/FSIS, MLG4.03, 2004. Isolation and Identification of *Salmonella* from Meat, Poultry and Egg Products 3: 1-16.