Isolation, Identification and Polymerase Chain Reaction (PCR) Detection of *Salmonella* Species from Field Materials of Poultry Origin

[1]Habtamu Taddele Menghistu, [2]Rajesh Rathore, [3]Kulip Dhama and [4]Rajesh Kumar Agarwal

[1]College of Veterinary Medicine, Mekelle University, P.O. Box: 1436, Tigray, Ethiopia
[2]Scientist, Centre for Animal Disease Research and Diagnosis (CADRAD),
Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.) - 243 122, India
[3]Senior Scientist, Avian Diseases Section, Division of Veterinary Pathology,
Indian Veterinary Research Institute, Izatnagar (U.P.) - 243 122, India
[4]Principal Scientist, Division of Bacteriology and Mycology,
Indian Veterinary Research Institute, Izatnagar (U.P.) - 243 122, India

Abstract: Poultry salmonellosis, one of the most prevalent diseases and major source of food-borne infections to humans due to consumption of poultry products is worldwide in distribution. The study was conducted from November 2008 to March 2009 with the aim of isolating *Salmonella* species by conventional culture method and their confirmation by Polymerase chain reaction (PCR). A total of 220 poultry tissue samples and 40 egg samples were processed during the study period for the isolation of *Salmonella* and an overall prevalence of 7/260 (2.7%) was found. The isolation of *Salmonella* from liver and intestine accounted for the highest among tissue samples processed. The remaining isolates were from spleen, pooled tissue samples and egg sample. According to serotyping result, three of the *Salmonella* isolates belong to *S.* Heidelberg which was the most predominant serotype in the present study. Other serotypes isolated include *S.* Typhimurium, *S.* Ayinde, *S.* Essen and *S.* Kastrup. The PCR amplification of suspected *Salmonella* isolates produced a product of approximate molecular size 550 bp and proved for the efficient utilization of this tool for the rapid detection of *Salmonella* organisms.

Key words: Isolation · PCR detection · Poultry · *Salmonella* · Serotypes

INTRODUCTION

Salmonellosis is a hyperendemic disease in India affecting both man and animals [1]. *Salmonella*, a family of Enterobacteriaceae, comprises 2541 serovars [2], distributed widely in nature and in India, more than 235 serovars (>53 from poultry birds alone) have been reported [3, 4] and this number is in constant increase. Contaminated poultry meat and eggs, particularly when the bacterium is present in the egg contents, are among the most important sources for food-borne outbreaks in humans and salmonellae are isolated more often from poultry and poultry products than from any other food animals [5-9]. Chickens can be infected with many different serovars of paratyphoid *Salmonella* [10]. Among these paratyphoid salmonellae, infections due to *S.* Typhimurium, *S.* Enteritidis and *S.* Heidelberg, are of

worldwide in distribution with wide host range and are of major economic and public health significance [11].

Conventional bacterial culture methods are still used most often to identify *Salmonella* and require at least 3-11 days [12-14]. The standard culture methods for detecting *Salmonella* spp. in poultry include non-selective pre-enrichment followed by selective enrichment and plating on selective and differential agars [15]. These methods are time consuming and labour intensive. The development of polymerase chain reaction (PCR) technology and real time PCR (RT PCR) have allowed the specific amplification of particular target segments of DNA [16, 17] which can be used for the detection of pathogens of veterinary importance. Recently, a lot of attention has been focused on PCR techniques to detect *Salmonella* as this is fast and reliable method for the rapid detection of this public health and animal associated

Corresponding Author: Habtamu Taddele Menghistu, College of Veterinary Medicine, Mekelle University,
P.O. Box: 1436, Tigray, Ethiopia. E-mail: habtamutaddele@yahoo.com.

pathogens. The objective of this study was to estimate the prevalence of *Salmonella* species in poultry tissue samples and confirmation by PCR detection.

MATERIALS AND METHODS

Sample Collection: From November 2008 to March 2009, a total of 220 random tissue samples of poultry was collected directly from the post mortem house of Indian Veterinary Research Institute (IVRI), Bareilly (U.P.), India. At necropsy, swabs were used to sample the poultry tissues particularly intestinal swabs and tissue samples were collected aseptically from the spleen, liver, kidneys, heart, lungs, digestive tract and ovary. In asymptomatic birds, large amounts of homogenized tissues were collected; in such cases pooled tissue samples from different birds have been performed. In addition, 40 Eggs were purchased randomly from the local markets and tested for the presence of *Salmonella*.

***Salmonella* Isolation and Identification Procedure:** The study was conducted utilizing the conventional methods for the detection of *Salmonella* following the standard guidelines from ISO 6579:2002 [18] with some modification (Microbiology of food and animal feeding stuffs horizontal method for the detection of *Salmonella* spp.). This isolation and identification procedure involved four principal stages: pre-enrichment, selective enrichment, selective plating and confirmation.

Five g of intestine, liver, kidney, heart and spleen was aseptically chopped in to fine pieces and added to 45 ml of buffered peptone water (BPW) (Oxoid, UK) and incubated at 37°C for 18 h. One ml of pre-enriched broth was transferred into tubes containing 10 ml selenite cystein broth (Himedia, India) and 10 ml tetrathionate brilliant green broth (Himedia, India) and further incubated at 37°C for 24 h. After incubation for 24 h, a loop-full of culture from each of the enriched broths was streaked onto plates of MacConkey agar, brilliant green phenol red lactose sucrose agar (BPLS agar) and hektoen enteric agar (HEA) (Himedia, India) [19]. The plates were incubated at 37°C for 24 h and checked for growth of typical colonies of *Salmonella* i.e. greenish blue colonies with a black centre on HEA and bright reddish and translucent colonies on BPLS agar. On MacConkey agar *Salmonella* colonies appear colourless and transparent. Further confirmation of suspected colonies was done by conventional biochemical methods [20- 22]. Biochemically confirmed *Salmonella* cultures were submitted to the National Salmonella Centre, IVRI, Izatnagar for further confirmation and serotyping (Fig. 1).

The isolation of *Salmonella* from egg samples involves the same procedure with the exception in the pre-enrichment step. The samples were mixed with a sterile spatula and then aseptically 5 ml of egg sample was taken from the mixture and added into 45 ml of sterile BPW. The inoculated samples were incubated at 37°C for 18 h.
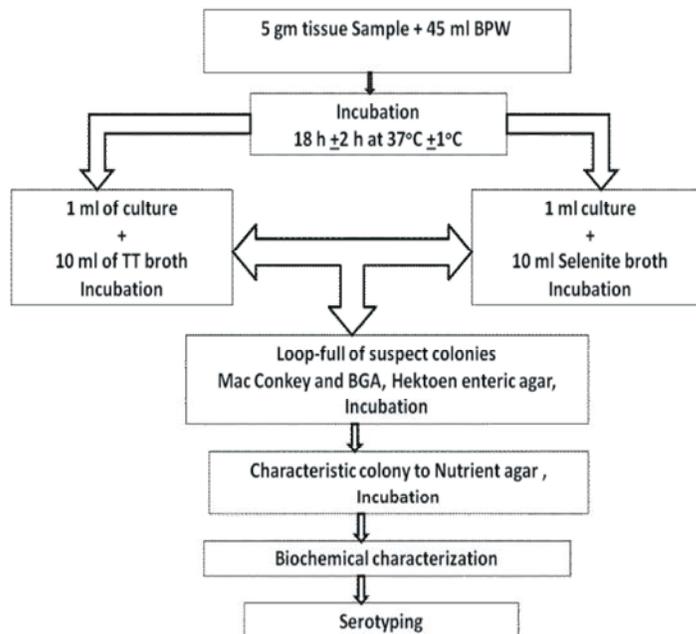

Fig. 1: Flow diagram showing procedure for isolation of Salmonella species [18]

Extraction of Genomic DNA: The genomic DNA was isolated following the method of Wilson [23] with slight modifications. A few colonies grown on selective agar was transferred into 10 ml Luria Berthani broth (Difco, France) and incubated at 37°C under constant shaking for 16 h. The bacterial cells were pelleted twice by centrifugation at 8000 rpm for 10 min at 4°C and then suspended in lysis buffer containing 567 µl TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA pH 8.0), 30 µl SDS (10% w/v) and 3 µl of proteinase K (20 mg/ml) followed by rapid pipetting and incubation at 37°C for 1 hr. The lysate was treated with 100 µl of 5M NaCl and 80µl Cetyl Trimethyl Ammonium Bromide (CTAB) (10%w/v in 0.7M NaCl w/v) with further incubation at 68°C in a water bath for 10 min. The aqueous phase was extracted twice with equal volume of phenol: chloroform (1:1) followed by extraction with equal volume of chloroform: isoamylalcohol (24:1). The supernatant was collected in a separate tube and DNA was precipitated by adding 1/10 volume of ammonium acetate (7.5 M) and double volume of chilled absolute ethanol. The DNA pellet obtained by centrifugation at 13,000 rpm for 20 min at 4°C was washed with 70% ethanol, dried and resuspended in 80 µl sterile triple distilled water and finally stored at -20°C in small aliquots.

Primers: The primers used were primer 1-forward (5'-GCA ACG CGA AGA ACC TTA CC-3') and primer 2- reverse (5'-GGT ACC TTG TTA CGA CTT T-3') (SBS Genetech, India) derived from 16s rRNA gene [24].

PCR Amplification: The components of the reaction mixture were optimized as follows: 100 ng of template DNA, 1x PCR assay buffer with (NH₄)₂SO₄, 2.5 mM MgCl₂ (MBI Fermentas, USA), each dNTPs at a concentration of 200µM, 1 U of *Taq* DNA polymerase (Banglore Genei, India) and 15 picomol solution of each primer (SBS Genetech, India), in 25 µl PCR reaction mix. PCR assay was performed in Gene Amp PCR System 9700 (Applied Biosystems, India) with a heated lid. The cycling conditions included 94°C for 5 min (predenaturation), 35 cycles of 94°C for 1 min (denaturation), 50°C for 45 sec (annealing) and 72°C for 1 min (polymerization) followed by 72°C for 10 min (final extension). The above PCR cycling conditions were chosen on the basis of preliminary experiments performed to optimise amplification of *Salmonella* target sequence. All amplifications included a blank, which contained all reagents but not the target DNA and reference strain of *S*. Gallinarum procured from the National Salmonella centre, IVRI, India as a positive control. The amplification products were checked for the presence of the desired bands on 1.2% agarose gel.

RESULTS

Prevalence of *Salmonella* During the Study Period: A total of 220 poultry tissue samples and 40 egg samples which was suspected for salmonellosis were collected from morbid material/post-mortem house and local markets respectively and processed during the study period. Out of these, 7 samples (2.7%) were found positive for *Salmonella* by conventional isolation and identification methods in the laboratory recommended by ISO 6579:2002. *Salmonella* was isolated from two liver and two intestine samples. The rest isolates were from spleen, pooled sample and eggs. The isolation of *Salmonella* from egg was 2.5% (1/40).

Fig. 2: Characteristic blue green with black centre colonies of Salmonella species on HEA plate (A) and Pinkish and transparent colonies of Salmonella on BPLS (B)
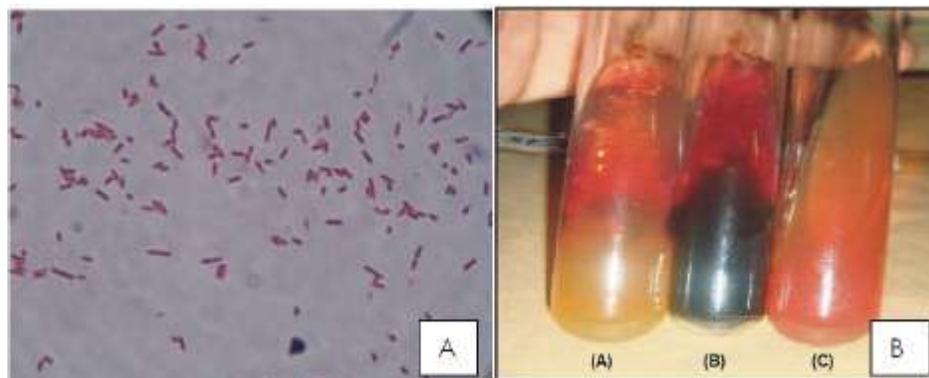
Fig. 3: Gram staining result of *Salmonella* taken from HEA plate (A) and TSI agar slants (B); A) Yellow butt showing only fermentation of glucose, B) Fermentation of glucose and H2S production (Black color) and C) Uninoculated TSI slant

Table 1: Summary of the biochemical test results of Salmonella isolates

	Sample type						
Biochemical test	Liver	Intestine	Intestine	Pooled sample	Spleen	Liver	Egg
TSI acid from glucose	+	+	+	+	+	+	+
TSI gas from glucose	+	+	+	-	+	+	+
TSI acid from lactose	-	-	-	-	-	-	-
TSI acid from sucrose	-	-	-	-	-	-	-
TSI H$_2$S production	+	+	+	+	+	+	-
Catalase	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-
Indole formation	-	-	-	-	-	-	-
Methyl red test	+	+	+	+	+	+	+
Voges Proskauer reaction	-	-	-	-	-	-	-
Citrate utilization	+	-	(+)	+	(+)	+	-
Urea hydrolysis	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+
Lysine decarboxylation	+	+	+	+	(+)	+	+
Phenylalanine deamination	-	-	-	-	-	-	-

(+): Positive after 48 h of incubation

Cultural and Morphological Characterization: On Mac Conkey agar plates all the *Salmonella* colonies appeared non-lactose fermenter, colourless and transparent. Six of the *Salmonella* isolates grown on HEA have shown typical blue green colonies with black dot at the centre (Fig. 2A) but one isolate was lacking the characteristic black dot at the centre. On BPLS agar the colonies of *Salmonella* test isolates were pinkish to red coloured and transparent (Fig. 2B). In Gram's staining, the morphology of the isolated bacteria was small rod shape, Gram negative, single or paired in arrangement (Fig. 3A). On TSI slants most of the *Salmonella* isolates showed fermentation of glucose, gas production from glucose, H$_2$S formation but none of the isolates fermented either lactose or sucrose (Fig. 3B).

Biochemical Characterization: All suspected colonies of *Salmonella* on the basis of cultural and morphological properties were subjected to selected biochemical tests including indole formation, methyl red and voges-proskauer reaction, citrate utilization, nitrate reduction, urea hydrolysis, triple sugar iron agar and phenyl alanine deamination. Out of the 11 suspected colonies, 7 were confirmed to be *Salmonella* species after the biochemical results. The seven *Salmonella* species were sent to the National *Salmonella* Centre, IVRI for further confirmation and serotyping. Table 1 summarises the biochemical test results of the *Salmonella* isolates recovered from poultry tissue samples and egg samples.

Table 2: Serotyping result of Salmonella isolated from poultry tissue samples and eggs

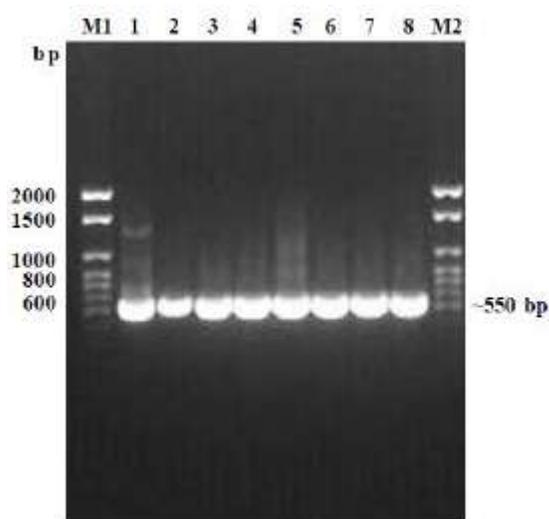| Sample type	Antigenic formula	Serotype
Liver	1, 4, 5,12: r: 1,2	*S. Heidelberg*
Spleen	1, 4, 5,12: r: 1,2	*S. Heidelberg*
Egg	1, 4, 5,12: r: 1,2	*S. Heidelberg*
Liver	4,5,12: i: 1,2	*S. Typhimurium*
Intestine	1,4,12,27: d: z6	*S. Ayinde*
Pooled sample	6,7: e,n,z15: 1,6	*S. Kastrup*
Intestine	4,12: gm: -	*S. Essen*

Fig. 4: PCR amplification of 16s rRNA gene loci (rrn) of Salmonella isolates
Lane M1 & M2: Molecular weight marker; Lane 1: *S.* Gallinarum; Lane 2: *S.* Heidelberg1; Lane 3: *S.* Heidelberg2; Lane 4: *S.* Heidelberg3; Lane 5: *S.*Typhimurium; Lane 6: *S.* Ayinde; Lane 7: *S.* Essen; Lane 8: *S.* Kastrup

Serotyping Result: The seven *Salmonella* isolates recovered during the study period were serotyped at the National Salmonella Centre, IVRI, Izatnagar. According to serotyping result three of the isolates were found to be *S.* Heidelberg and the remaining four were *S.* Typhimurium, *S.* Essen, *S.* Ayinde and *S.* Kastrup as shown in Table 2.

PCR Detection of *Salmonella* Species: All the *Salmonella* suspected cultures subjected to PCR amplification generated a product of approximate molecular size 550 bp. 100 bp DNA marker (MBI Fermentas, USA) was used as a molecular weight marker. The band size detected in all the *Salmonella* isolates and *S.* Gallinarum reference strain was consistent as analysed by agarose gel electrophoresis (Fig. 4).

DISCUSSION

Isolation of *Salmonella* from poultry and poultry products is higher compared to the isolation from other animal species [9, 25]. Therefore, poultry and their products are widely acknowledged as the major sources of food borne salmonellosis to human beings. In the present study an overall prevalence of *Salmonella* isolation from poultry tissue samples was 2.7% which is of economic and public health significance for the country. Isolation of *Salmonella* from the field tissue samples revealed identification of two isolates from liver and intestine each and one isolate each from spleen and pooled tissue samples.

The findings of this study in relation to contamination of poultry tissue samples with *Salmonella* is lower than those observed by other authors, as 11.4% [26] and 5.36% [27]. This variation may be associated with different factors such as, season of the study, geographic location, number of samples and hygienic conditions in the farm. Similarly, *Salmonella* contamination of eggs observed in this study is somewhat lower than the observation by other authors in the country [28, 29]. This variation may also be due to the same reasons as mentioned above for tissue samples. Isolation of *Salmonella* from liver and intestine was relatively higher among tissue samples processed and this finding is in close agreement with previous reports [27, 30-32]. Hossain *et al.* [26] and Sujatha *et al.* [33] have also indicated higher rate of isolation of *Salmonella* from liver and ovary samples.

All the *Salmonella* serotypes isolated in this study belong to the paratyphoid *Salmonella* with the most predominant serotype being *S.* Heidelberg. This serotype is one of the most commonly identified serotype in chickens next to *S.* Enteritidis and *S.* Typhimurium [34]. Even in non-clinical cases *S.* Heidelberg may account for the highest rate of isolation than the most common types, *S.* Enteritidis and *S.* Typhimurium [10, 35]. Since the mid 1990s, *S.* Heidelberg is becoming an important pathogen

of the poultry industry and this pathogen has been shown to colonize the reproductive tract and enter eggs, similar to what is observed with *S*. Enteritidis [36, 37]. Serologically, *S*. Typhimurium, *S*. Heidelberg, *S*. Ayinde and *S*. Essen are members of serogroup B (O:4) and *S*. Kastrup belongs to serogroup C1 (O:7). Serotypes, *S*. Ayinde, *S*. Essen and *S*. Kastrup are rarely isolated from poultry and are of less economic and public health significance.

In the present study, specific biochemical media were used for the detection of *Salmonella*. All of the isolates fermented glucose but did not ferment lactose and sucrose in TSI slants and all of the isolates were indole negative, methyl red positive, VP negative and urease negative which are special biochemical characters for *Salmonella* species as previously suggested by other workers [38, 39]. In majority of the isolates gas was produced from glucose and H_2S was also produced. In this study, the colony characters of *Salmonella,* the production of hydrogen sulfide with black colour colonies on HEA and typical pinkish and transparent colonies on BPLS agar was in accordance with the facts in the literature [40]. In Gram's staining, the morphology of the isolated bacteria showed small rod shape, Gram negative, single or paired in arrangement which was in agreement with standard morphological characters of the organism as described by several authors [22].

Repeated outbreaks and higher incidence of salmonellosis in industrialized as well as developing countries over the past decades [41- 43] necessitates for the development of rapid and accurate detection methods for *Salmonella* species, since the conventional methods for the isolation and identification of salmonellae require up to 3-11 days [13]. Recently, the development of PCR targeting specific gene has become a powerful and increasingly popular tool in detection and confirmation of pathogens of veterinary importance [44]. The primers used in this study, Primer 1 and Primer 2, were proved to be specific for the PCR detection of all *Salmonella* isolates with various serotypes as all *Salmonella* isolates identified by conventional tests gave positive bands with PCR.

In conclusion, this study showed for the presence of *Salmonella* species among poultry tissue samples and eggs which is of economic and public health significance for the country and the high frequency of the *Salmonella* Heidelberg isolates magnifies the public health significance as this is most important zoonotic *Salmonella* species next to *S*. Typhimurium and *S*. Enteritidis. But the conventional isolation methods are time consuming and labour intensive and may have low sensitivity for the identification of samples with low initial numbers of *Salmonella*, as may often be seen in sub-clinically infected chickens, resulting in false-negative test results. Therefore, the application of PCR for the rapid detection of *Salmonella* species is a promising tool and it can also be applied to direct clinical materials as it is highly sensitive test.

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