P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(3): 3586-3590
© 2018 IJCS
Received: 15-03-2018
Accepted: 18-04-2018

Surendra Singh Shekhawat
Assistant Professor, Dept. of Veterinary Public Health, CVAS, Navania, Udaipur, Rajasthan, India

Abhishek Gaurav
Assistant Professor, Dept. of Veterinary Public Health, CVAS, Navania, Udaipur, Rajasthan, India

Bincy Joseph
Assistant Professor, Dept. of Veterinary Microbiology, CVAS, Navania, Udaipur, Rajasthan, India

Shiv Kumar Sharma
Associate Professor, Dept. of Veterinary Medicine, CVAS, Navania, Udaipur, Rajasthan, India

Rohitash Dadhich
Associate Professor, Dept. of Veterinary Pathology, PGIVER, Jaipur, Rajasthan, India

CS Vaishnava
Professor, Department of Animal Nutrition, CVAS, Navania, Udaipur, Rajasthan, India

Correspondence
Surendra Singh Shekhawat
Assistant Professor, Dept. of Veterinary Public Health, CVAS, Navania, Udaipur, Rajasthan, India

Molecular detection of *Salmonella enterica subspecies enterica* serotypes in foods of animal origin by PCR

Surendra Singh Shekhawat, Abhishek Gaurav, Bincy Joseph, Shiv Kumar Sharma, Rohitash Dadhich and CS Vaishnava

Abstract
This study was aimed at identifying *Salmonella enterica sub species enterica* serotypes by amplification of salmonella genus specific 16S ribosomal RNA gene (*16SrRNA*) in foods of animal origin in and around Udaipur city. Food samples (total, n=632) of animal origin i.e. meat (n=206) milk (n=215) and egg (n=211) were purchased randomly from various retails shops located in the study area. Of the 632 samples, 10 isolates (1.58%) were positive for salmonella by conventional culture method specified by IS 5887 (Part 3):1999. DNA isolated from all the ten isolates was followed by molecular confirmation by polymerase chain reaction of *16SrRNA* gene. The PCR products were subjected to agarose gel electrophoresis and all the ten isolates were found to be positive for *16SrRNA* gene. This shows that PCR can be used as an alternative to the conventional methods of salmonella detection in food samples. The study showed that *16SrRNA* primers are highly specific and sensitive for salmonella detection and the results can be obtained within 4-5 hours' time. *16sRNA* based PCR will be rapid and effective method for the surveillance of salmonella in foods of animal origin. Thus PCR offer a rapid and good diagnostic tool for routine monitoring of salmonellae in food samples compared to the conventional culture method.

Keywords: *16SrRNA*, udaipur, PCR, meat, milk, egg

Introduction
Foodborne illnesses are defined by the world health organization as diseases of infectious or toxic nature caused by consumption of contaminated foods or water. The burden of foodborne diseases is substantial: every year almost 1 in 10 people fall ill and 33 million of healthy life years are lost. Diarrhoeal diseases are the most common illnesses resulting from unsafe food, 550 million people falling ill each year, including 220 million children under the age of 5 years. Salmonella is one of the 4 key global causes of diarrhoeal diseases. The gravity of the disease can be best contemplated by the fact that between 1996 and 2006 more than 50,000 confirmed cases of nontyphoidal salmonella infections have been reported annually in U.S, an average of 4.7 infections per 10,000 persons per year (Tsai, *et al.* (2007) [30]. Jones *et al*. 2008) [13]. In 2009 among 17,468 laboratory-confirmed cases of infections identified by food borne diseases active surveillance network, most were salmonella (CDC (2010) [6]. An estimated 93.8 million cases (90% CI, 61.8-131.6 million) of gastroenteritis caused by *Salmonella* species occur globally each year and of these, nearly 80.3 million cases are foodborne (Majowicz *et al.,* 2010) [16].Salmonella needs special concern in developing world because of the poor hygienic conditions that favor it's spread (Nagappa *et al.* (2007) [18] and also due to low socio-economic status.

Among various pathogens causing foodborne illness salmonella is one of the most serious pathogen and is responsible for numerous food borne outbreaks reported worldwide (Thakur and Bajaj(2006) [28]. Salmonella, a member of family *Enterobacteriaceae* with 2579 serovars (Grimont and Weill (2007) [10] is rod-shaped, Gram-negative bacteria and most of them are considered as human or animal pathogens. *Salmonella* relies on multiple virulence factors, many of which are clustered within *Salmonella* pathogenicity islands (SPIs) to cause a disease, and a total of 19 SPI have been described with SPI-1 to SP-5 being present in most serovars and others were being less widely distributed (Foley *et al*. (2008)[8]. The 16SribosomalRNA (*16SrRNA*) is one of the *Salmonella* pathogenicity island I (SPI-I) virulence genes (Ziemer and Steadham (2003) [33].

The use of *16SrRNA* gene sequencing within the regulatory workflow May help to reduce the time and labor involved in the identification and differentiation of *Salmonella enterica* isolates.

Strains in *Salmonella enterica subspecies enterica* serogroup I cause approximately 99 percent of salmonella infections in humans and warm blooded animals (Popoff and Le Minor (1997) [20]. Salmonella infections in human beings are mostly from contaminated foods as pork, beef, poultry meat, egg, milk, milk products and contaminated water. Among these contaminated poultry, meat and eggs are the most frequently implicated sources of outbreaks of human salmonellosis (Breytenbach (2004) [4]. So, the food safety and regular screening of foods of animal origin is atmost important to avoid salmonella infection in human beings. Eventhough conventional methods of isolation and identication of Salmonella from food sample is perfect it is tedious and time consuming.

Nowadays, these conventional methods are replaced by most rapid and reliable PCR techniques. Several PCR-based assays have been developed for rapid detection of *Salmonella spp*. (Rahn *et al*. (1992) [22]. Dobhal *et al*. (2014) [7]. PCR of 16S ribosomal RNA (*16SrRNA*) gene can be used to detect and discriminate between *Salmonella* and non-Salmonella species (Ziemer and Steadham (2003) [33] and when coupled with standard microbiological procedure, it is a rapid and sensitive technique for the detection of salmonella (Lin and Tsen (1996) [15].

Materials & Methods

Samples

A total of 632 food samples of animal origin (meat, n=206; egg, n=211 and milk,n=215) were collected randomly from markets during a period of one year from March, 2017 to February,2018. Meat, egg & raw milk samples were obtained from various retail shops and dairies located in and around Udaipur city, Rajasthan. The samples were transported under complete aseptic conditions in an icebox within 2 h to the Department of Veterinary Public Health, CVAS, Navania (Udaipur). The samples were analyzed immediately after the receipt of the samples.

Isolation and biochemical characterization *Salmonella species*

Processing of meat, egg and milk samples for isolation for *Salmonella spp.* was done according to IS 5887 (Part 3) [12]: 1999. Briefly, each of 25 g meat sample was cut into fine pieces where as 25 ml each of egg and milk samples were homogenized separately with 225 ml of Buffered Peptone Water (BPW) (HiMedia, India) in a sterile polythene bag to obtain 1 part sample + 9 part Buffered Peptone Water and was shaken approximately for 2 min followed by incubation at 37^0C$\pm$ 1^oC for 24 hours for pre-enrichment. Following incubation, 0.1 ml of inoculum from this pre-enrichment was transferred into 10 ml of Rappaport-Vassiliadis (RV) medium (Hi-media, Mumbai) for enrichment and further incubated at 42^0 C for 24 hr. Then a loopful of the inoculum from RV broth was streaked on Xylose Lysine Deoxycholate (XLD) agar. The plates were incubated at 37^0 C $\pm$ 1^0 C for 24 hr for the development of growth of *Salmonella spp*. Characteristic pink or red colonies with or without black centre on XLD agar were characterized biochemically by Indole (I), Methyl Red (MR), Voges Proskeur (VP), Citrate(C),Triple Sugar Iron(TSI) and Urease test. The results were interpreted and validated as per Bacteriological Analytical Manual for *Salmonella* (2007) [1]. The colonies showing Salmonella specific IMViC pattern (-+-+) were inoculated on TSI slant. Slants with characteristic TSI reactions were further tested for urease enzyme production by inoculating urea agar slant. All the urease negative isolates were considered as biochemically confirmed and further subjected to molecular characterization.

Molecular Characterization

DNA isolation

DNA isolated from all the ten biochemically confirmed isolates using HiMedia TM Bacterial Genomic DNA Purification Kit following the manufacturer's instructions supplied along with the kit with suitable modifications. Briefly, 1.5 ml of overnight broth culture was pelleted by centrifugation at 15,000 rpm for 2 minutes. The supernatant was discarded and pellet was resuspended in 180 µl of lysis solution AL. After adding 200 µl lysis solution C_1 it was vortexed for 15 seconds and then incubated at 55⁰ C for 10 minutes. Then 200 µl of ethanol (95-100%) was added to the lysate and mixed thoroughly by vortexing for 15 seconds. The lysate so obtained was transferred into Spin Column and centrifuged at 10,000 rpm for 1 minute. The flow-through liquid was discarded and placed in a new 2 ml collection tube. Then 500 µl of prewash solution was added to the spin column and centrifuged at 10,000 rpm for 1 minute. The flow through was again discarded and same collection tube was used. A volume of 500 µl of diluted wash solution was added to column and centrifuged at 15,000 rpm for 3 minutes and spin again at same speed for the additional 1 minute to dry the column. The HiElute spin column was placed on fresh tube and 100 µl of elution buffer which was kept in water bath at 65^0 C for 30 minutes. The column was incubated at room temperature for 5 minutes followed by centrifugation at 10000 rpm for 1 minute. The spin column was then removed and the collected DNA was stored at -20⁰ C for further use.

The concentration of DNA isolated was estimated spectrophotometrically in Biospectrometer,(eppendorf, USA) using following formula.

DNA concentration (µg/ µl) = [OD_{260} x dilution factor x 50 mg/ml]/1000

The purity was checked as ratio of OD_{260} and OD_{280} and the integrity of the purified DNA was assessed by running it in 0.7% agarose gel.

Polymerase chain reaction of *16SrRNA*

Genomic DNA isolated from salmonella isolates were used in the PCR. Published primers targeting genus specific region of *16SrRNA* gene of *Salmonella enteric subspecies enterica* by Lin and Tsen (1996) [15] were used in the present study. The details of the primer is given in table 1. PCR was conducted in 25 µl reaction volume containing 1 µl of genomic DNA from the salmonella isolates, 10.5µl master mix, 0.5 µl of primers specific for *16SrRNA* and 10.5 µl of nuclease free water. The cycling conditions for amplification were as follows; initial denaturation at 95ºC for 5 mins followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 42ºC for 30 sec and extension at 72°C for 30 sec. followed by a final extension step at 72°C for 10 minutes. The amplified products were electrophoresed for the presence of desired bands on 0.9 % agarose gel and visualized and documented in a UV transilluminator. A 100 bp DNA ladder & 1 kb ladder (Thermoscientific, Lithuania) were used as markers for determining the molecular weight of PCR products (Nair *et al.* (2002) [19].

Table 1: Details of the primers used in the present study

Primer	Sequence	Expected product size	Tm value
16S rRNA (forward)	(5' TGT TGT GGT TAA TAA CCG CA 3')	574 bp	47.68
16S rRNA (reverse)	(5' CAC AAA TCC ATC TCT GGA 3')		45.77

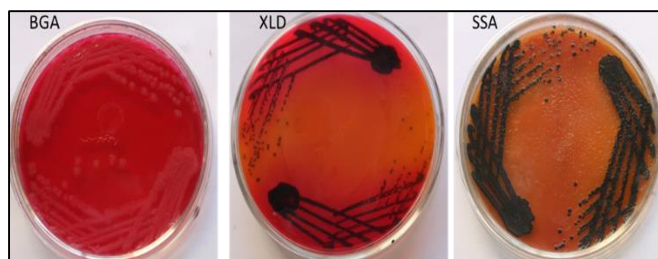
Results and Discussion

Salmonella is considered as the most prevalent foodborne pathogen worldwide (Sánchez-Vargas *et al.* (2011) [24]. Carasso *et al*. (2012) [5] has long been recognized as an important zoonotic microorganism of economic significance in animals and humans predominantly in the developing countries. Consumption of raw or unsafe food, cross-contamination, improper food storage, poor personal hygiene practices, inadequate cooling and reheating of food items, and a prolonged time lapse between preparing and consuming food items were mentioned as contributing factors to an outbreak of salmonellosis in humans (Käferstein (2003) [14]. Carasso *et al.* (2012) [5]. The ubiquity of *Salmonella* isolates creates a persistent contamination hazard in all raw foods and also in animal-origin food products, which are often implicated in sporadic cases and outbreaks of human salmonellosis (Macowiz *et al*. (2010) [16]. Tadesse (2014) [26]. Foodborne transmission is recognized as the major cause of *Salmonella* infections, with many food sources and supplies implicated in these infections (Majowicz *et al.* (2010) [16]. So it is very important to regularly screen the food items especially the foods of animal origin to study the prevalence of salmonellosis in human and animal population of a region. Conventional bacterial culture methods are still used most commonly to identify Salmonella and require at least 3-11 days (Tate *et al.* (1992) [27]. Wallece *et al.* (1999) [31]. These 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, there is a need for the development of rapid and accurate detection methods for *Salmonella* species as these organisms are causing severe economic losses as a result of infections of humans, animals and birds.

The rapid, cost-effective, and automated diagnosis of food-borne pathogens throughout the food chain continues to be a major concern for the industry and public health. Because of these requirements, the PCR became a powerful tool in microbiological diagnostics during the last decade (Sachse(2003) [23]. Polymerase chain reaction allows the detection of *Salmonella species* in food within a maximum of 12 h from the receipt of food samples. In the present study, ten salmonella isolates were obtained from the 632 (1.58%) samples screened. Then all the isolates were found to be positive for salmonella by cultural (fig.1) and biochemical methods(Fig 2). But the results of cultural and biochemical test will be always ambiguous as the organism shares most of cultural and biochemical properties with the genus proteus in the Family Enterobacteriaceae. But the PCR targeting the 16srRNA gene have excellent discriminative power to discriminate salmonella from proteus. (Lin and Tsen. (1996) [15] *16SrRNA genus* specific sequence can be used for the detection of *Salmonella* in food samples. All the ten isolates which are confirmed by biochemical test showed amplification of *16SrRNA* gene (574bp) using the primer set *16SrRNA*(F-5' TGT TGT GGT TAA TAA CCG CA 3' and R- 5' CAC AAA TCC ATC TCT GGA 3') Fig 3.

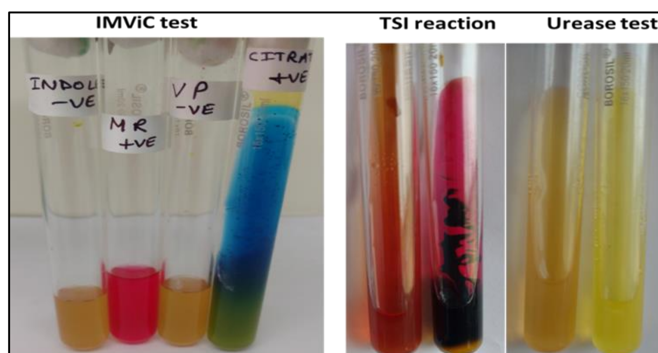
Previous studies have also shown that this PCR is rapid and reproducible in identification of isolates (Gallegos-Robles *et al.* 2008) [9]. These results showed the amplification of *16SrRNA* can potentially be applied for identification of *Salmonella spp.* Various primers sets targeting Salmonella *16SrRNA* genes are already available (Hellberg *et al*. 2012) [11]. Recent study on the molecular epidemiology and in vitro antimicrobial susceptibility of Salmonella isolated from poultry in Kashmir valley, India showed that all the isolates of *Salmonella* were tested by genus-specific polymerase chain reaction (PCR), using the 16S ribosomal RNA (rRNA) primers (Mir *et al.* (2010) [17].

In India, salmonellosis is endemic and its importance as potential zoonosis needs no emphasis as it causes heavy economic losses every year (Rahman (2002) [21]. *Salmonella*, a major public health problem in India is noted to be in increasing trend (Bhattacharya and Dash (2007) [3]. Shahane *et al.* (2007) [25]. Proper characterization of Salmonella isolates is essential for investigations of Salmonella outbreaks as non-typhoidal Salmonella strains have reached epidemic proportions in many countries despite improvements in sanitation and hygiene (Threlfall and Frost (1990) [29]. In view of limited adherence to high standards of hygiene, the probability of bacterial contamination of food at various stages of processing is always very high (Benslam *et al.* (2010) [2]. Regular screening and monitoring of food at various stages of food processing is very essential to avoid food borne infections. The study showed that 16sRNA based PCR will be rapid and effective method for the surveillance of salmonella in foods of animal origin. Thus PCR offer a rapid and good diagnostic tool for routine monitoring of salmonella in food samples compared to the conventional culture method.



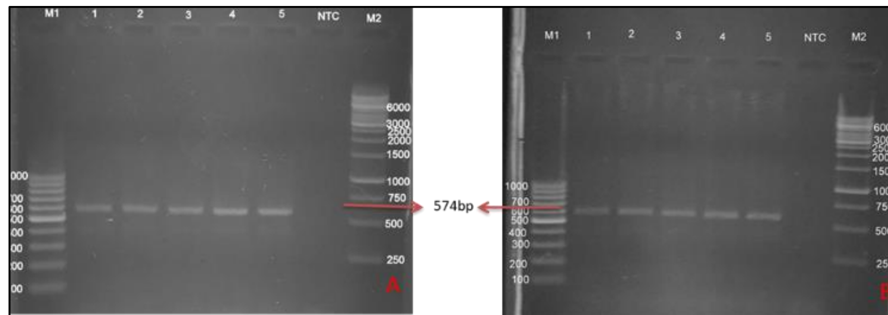
* BGA: Brilliant Green Agar, **XLD: Xylose Lysine Deoxycholate Agar, ***SSA: Salmonella Shigella Agar

Fig 1: Cultural characteristic of isolates on different selective media for salmonella



* IMViC reaction for Slamonella is: -/+/-/+ ** TSI reaction:Acid butt, alkaline slant, H_2S production *** Urease negative

Fig 3: PCR amplification of 574 bp of 16SrRNA gene using Salmonella genus specifc primers.



M1 is 100bp ladder M2 is 1 kb ladder in Plate A & Plate B.
L1 to L5 in Plate A are isolates S98, S115, S158, S221, S306
L1 to L5 in Plate B are isolates S410, S453, S485, S522, S570

Fig 3: PCR amplification of 574 bp of 16SrRNA gene using Salmonella genus specifc primers

Conclusion

In the present study, prevalence of salmonella was found to be 1.58% in the foods of animal origin in the study area. Based on the methods and results of the present study we can conclude that for the surveillance and monitoring of salmonella in food samples the PCR prove to be a rapid efficient and sensitive technique as compared to conventional culture methods.

References

1. Bacteriological Analytical Manual. BAM: *Salmonella*, Chapter 5 *Salmonella*. http://www.fda.gov/ downloads/Food/Food Science Research/UCM309839, 2007.
2. Ben Salem I, Aouni M, Mzoughi R. Specific detection of Salmonella spp in food by multiplex Polymerase Chain Reaction. Advance Studies in Biology. 2010; 2(2):73-88.
3. Bhattacharya SS, Dash UA. Sudden rise in occurrence of *Salmonella* Paratyphi A infection in Rourkela, Orissa. Indian J Med Microbiol. 2007; 25:78-79.
4. Breytenbach JH. Salmonella Control in Poultr.Intervet International b.v, 2004, 1-4.
5. Carrasco E, Morales-Rueda A, García-Gimeno RM. Cross-contamination and recontamination by *Salmonella* in foods: a review. Food Research International. 2012; 45(2):545-556.
6. Centers for Disease Control (CDC) Foodborne Diseases Active Surveillance Network (Food Net), United States, MMWR Morb Mortal Wkly Rep. 2010, 59(40).
7. Dobhal S, Zhang G, Rohla C, Smith MW, Ma LM. A simple, rapid, cost-effective, and sensitive method for detection of Salmonella in environmental and pecan samples. J Appl Microbiol. 2014; 117(4):1181-90.
8. Foley SL, Lynne AM and Nayak R. *Salmonella* challenges: Prevalence in swine and poultry and potential pathogenicity of such isolates. J Anim. Sci., 2008; 86:E149-E162.
9. Gallegos-Robles AM, Morales-Loredo A, Alvarez-Ojeda G, Vega-PA, Chew-MY, Velarde S, *et al*. Identification of Salmonella Serotypes isolated from cantaloupe and chile pepper production systems in Mexico by PCR–Restriction Fragment Length Polymorphism, Journal of Food Protection. 2008; 71:2217-2222.
10. Grimont PAD, Weill FX. Antigenic formula of Salmonella seovars. 9th ed. WHO Collaborating Centre for reference and Research on Salmonella, Institute Pasteur, France, 2007.
11. Hellberg DR, Haney JC, Shen Y, Cheng MC, Williams-Hill MD, Martin BM. Development of a custom 16SrRNA gene library for the identification and molecular subtyping of Salmonella enteric.Journal of Microbiology Methods 2012; 91(3):448-458.
12. IS 5887. Methods for detection of bacteria responsible for food poisoning Part 3: General guidance on methods for the detection of Salmonella, 1999.
13. Jones TF, Ingram LA, Cieslak PR. Salmonellosis outcomes differ substantially by serotype. J Infect Dis. 2008; 198 (1):109-14.
14. Käferstein F. Foodborne diseases in developing countries: aetiology, epidemiology and strategies for prevention. International Journal of Environmental Health Research. 2003; 13(1):S161-S168.
15. Lin C, Tsen H. Use of two 16S DNA targeted oligonucleotides as PCR primers for the specific detection of salmonella in foods. Journel of Applied Bacterilogy. 1996; 80:659-666.
16. Majowicz SE, Musto J, Scallan E, Angulo FJ, O'Brein SJ, Jones TF. The global burden of nontyphoidal salmonella gastroenteritis. Clin. Infect. Dis. 2010; 50(6):882-889.
17. Mir I, Wani S, Hussain I, Qureshi DS, Bhat AM, Nishikawa Y. Molecular epidemiology and in vitro antimicrobial susceptibility of Salmonella isolated from poultry in Kashmir. Revue Scientifque et Technique de l'Offi ce International des Epizooties. 2010; 29(3):677-686.
18. Nagappa K, Tamuly S, Brajmadhuri, Saxena MK, Singh SP. Isolation of *Salmonella* Typhimurium from poultry eggs and meat of Tarai region of Uttaranchal. Indian Journal of Biotechnology. 2007; 6:407-409.
19. Nair S, Kwai Lin T, Pang T, Altwegg M. Characterization of salmonella serovars by PCR-single-strand conformation polymorphism analysis. Journa of Clinical Microbiology. 2002; 40:2346-2351
20. Popoff MY, Minor LLe. Antigenic formulaa of the Salmonella serovars, 7th revision. World Health Orgnization Collaborating Centre for Reference and Research on Salmonella Pasteur Institute, Paris, France. 1997.
21. Rahman H. Some aspects of molecular epidemiology and characterisation of Salmonella typhimurium isolated from man and animals. Indian J Med. Res. 2002; 115:108-112.
22. Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galan´ JE, Ginocchio C *et al.* Amplification of an invA gene sequence of Salmonella Typhimurium by polymerase chain reaction as a spe-cific method of detection of Salmonella. Mol Cell Probes. 1992; 6(4):271-9.
23. Sachse K. Specificity and performance of diagnostic PCR assays. Methods Mol. Biol. 2003; 216:3-29.

24. Sánchez-Vargas FM, Abu-El-Haija MA, Gómez-Duarte OG. *Salmonella* infections: an update on epidemiology, management, and prevention. Travel Medicine and Infectious Disease. 2011; 9(6):263-277.
25. Shahane V, Muley V, Kagal A, Bharadwaj R. Non-typhoid Salmonellosis: Emerging infection in Pune. Indian J Med Microbiol. 2007; 25:173-174.
26. Tadesse G. Prevalence of human Salmonellosis in Ethiopia: a systematic review and meta-analysis. BMC Infectious Diseases. 2014;14, article no. 88 doi: 10.1186/1471-2334-14-88.
27. Tate CR, Miller RG, Mallinson ET. Evaluation of two isolation and two nonisolation methods for detecting naturally occurring Salmonellae form broiler flock environmental drag-swab samples. J Food Prot. 1992**;** 55**:** 964-967.
28. Thakur YR, Bajaj BK. Antibiotic resistance and molecular characterization of poultry isolates of salmonella by RAPD-PCR. World Journal of Microbiology and Biotechnology. 2006; 22(11):1177-1183.
29. Threlfall JE, Frost AJ. The identification, typing, and fingerprinting of Salmonella; laboratory aspects and epidemiological applications. Journal of Applied Bacteriology. 1990; 68:5-16.
30. Tsai MH, Huang YC, Chiu CH. Nontyphoidal salmonella bacteremia in previously healthy children: analysis of 199 episodes. Pediatr Infect Dis J. 2007; 26(10):909-13.
31. Wallace HA, June G, Sherrod P, Hammack TS, Amaguana, RM. *Salmonella*. *In*: Food and Drug Administration bacteriological analytical manual.1999.
32. Withee J, Dearfield KL. Genomics-based food-borne pathogen testing and diagnostics: possibilities for the U.S. Department of Agriculture's Food Safety and Inspection Service. Environ Mol Mutagen. 2007; 48:363-8.
33. Ziemer C J, Steadham RS. Evaluation of the specificity of Salmonella PCR primers using various intestinal bacterial species. Letters in Applied Microbiology. 2003; 37:463-469.