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Full Length Research Paper

Simple and rapid detection of *Salmonella sp*. from cattle feces using polymerase chain reaction (PCR) in Iran

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The aim of this study was to employ biochemical and molecular assays to detect and diagnose *Salmonella* in cattle. For this reason, 1124 fecal samples were collected from cattle in Markazi provinces of Iran. Selective specific culture media for *Salmonella* were used to grow a number of isolates from the cattle samples. *Salmonella* bacteria were identified with biochemical test. The antimicrobial susceptibility test with disc diffusion method was performed on samples of *Salmonella* by using a molecular based approach, and it was possible to identify *Salmonella sp* by amplifying specific genes *''16s rRNA''* as a step for identification. Our studies showed that the molecular-based approach are more rapid for initial detection of *Salmonella SP*.

Key words: Salmonella, cattle, detection, polymerase chain reaction (PCR), 16s rRNA.

INTRODUCTION

The genus *Salmonella* consists of over 2668 different serotypes (Alena and Mark, 2009). Salmonellosis is responsible for large numbers of infections in both humans and animals (Keusch, 2002). *Salmonella* strains are not detectable in certain clinical samples that contain small numbers of organisms (Fricker, 1987). However, the number of *salmonella* present in the faeces of an infected individual is large, that is, approx. This level of excretion is maintained for several weeks, before falling gradually until the individual no longer excretes (Taylor and McCoy, 1969). Furthermore, after the disappearance of the organism from the intestinal tract, up to 5% of patients, upon recovery from this disease, may become carriers who shed the organism in their faeces (Jay, 2000). Therefore, detection of *Salmonella* strains in faecal samples is not only important for the diagnosis of salmonellosis, but also essential to identify carriers of this organism, especially among food handlers, who have higher risks of spreading the pathogen.

Majority of the human salmonellosis cases are caused by consumption of contaminated egg, poultry, pork, beef and milk products (Geimba et al., 2004). *Salmonella* infections in calves continue to be a major problem worldwide. Substantial economical losses were manifested through mortality and poor growth of infected animals as well as the hazard of transmitting food poisoning to humans. *S. typhimurium*, *S. enteritidis*, *S. anatum S. newport*, *S. cerro*, *S. montevideo*, *S. agona* and *S. dublin* was considered the major host-adapted Salmonella from cattle (Mitz et al., 1981; Konrad et al., 1994; Ritchie et al., 2001; Veling et al., 2002). Typhimurium is the most common serovar isolated from diarrheal patients, and Choleraesuis, Dublin, and Enteritidis are often isolated from patients with bacteremia (Guiney, 1995).

Salmonella has been widely reported in cattle (Field,

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Table 1. primer information: sequence (Seq), optical density (OD), molecular weight (MW), and temperature melting (TM).

Primer name	Sequence (seq)	OD	MW	100PM/ µL	TM
Forward	5´→ 3´ AGAGTTTGATCATGGCTCAG	3.1	6172	138	55.3
Reverse	5 →´3´ GGTTACCTTGTTACGACTT	3/1	5784	158	52.4

Table 2. Rate of isolated *Salmonella* sp from fecal cattle in Arak province in Iran.

Samples	Positive samples	Positive samples (%)	Negative samples	Negative samples (%)
1124	36	3.2	1088	96.79

1948; Hughes et al., 1971; Wray et al., 1977; Hollinger et al., 1998; McDonough et al., 1999). The infected animals may shed the organism in their feces without showing any clinical signs of disease (Gibson, 1965). Thus a rapid, specific and sensitive detection method for Salmonella is important for animal and human health and for the diagnostic industry (Gouws, 1998). In this pilot study, we analyzed the *16S rRNA* sequences of 36 isolates of 1124 cattle samples that have been isolated in Arak province of Iran. Our goal was to establish a simple and rapid sequence-based method for molecular identification.

MATERIALS AND METHODS

Samples

1124 fecal samples were randomly collected from 15 different farms since one month age and above, and collected during several months.

Isolation *salmonella*

Fecal samples were placed in enrichment medium and then transported to Razi Vaccination and Serum Research Institute. The samples were cultivated on to selective medium such as SS agar for 18-24 h at 37°C. For identification of *salmonella* colonies, samples were subjected to biochemical tests such as Triple sugar iron (TSI), Sulfide-Indole-Motility medium (SIM), (Methyl Red, Voges-Proskauer (MRVP), Urea, and Catalase and finally reconfirmed as negative-bacilli or coco bacilli by optic microscope.

Antibiogram test

The antimicrobial susceptibility testing with disc diffusion method was performed on samples of salmonella. The test was evaluated in *Salmonella* susceptibility to 16 antibiotics Including Lincospectin, Enrofloxacin, Tobramycin, Nitrofurantoin, Imipenem, Gentamycin, Doxycycline,Co-trimoxazole, Ciprofloxacin, Chloramphenicol, Cephalothin, Ceftriaxone, Cefotaxime, Cefazolin, Ampicillin, Amikacin

Chromosomal DNA extraction

Salmonella isolates were cultivated on Luria Bertani (LB) for 18-24 h at 37°C; the extraction of DNA was performed according to the method of Sambrook (2000).

Primers

Two universal oligonucleotides primers mentioned in Table 1 were obtained from fermentas (USA). The primers were used to amplify the sequences of 16s rRNA.

Polymerase chain reaction (PCR)

Amplification program was carried out as described previously, PCR was done in 25 µL reaction volumes, 2 µL of 10X PCR buffer, 1 µL of $MgCl_2$, 1 µL of 10 mM dNTP, 0/5 µL of Taq DNA Polymerase (Fermentase), 1 µl from each primer (Cinnagen), 3 µL of sample. The reaction was completed up to 25 µL with distilled water. The PCR was programed to 2 min for denaturation at 95°C, 34 cycles to denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extraction at 72°C for 1 min following by 72°C for 10 min. Then stored at -20°C (Hosseini et al., 2003).

Electrophoresis of PCR products

The amplified DNA products from Salmonella spp specific-PCR were analyzed with electrophoresis on 1% agarose gels stained with ethidium bromide and visualized by UV illumination.

RESULTS AND DISCUSSION

36 samples out of 1124 samples from cattle feces were isolated as positive. In biochemical test, isolated *salmonella sp* were lactose (-), indol (-), urea (-), catalase (+), and TSI test was K/A. The entire positives were confirmed by using coloring gram and optical microscope as Gram negative bacilli and coco bacilli (Table 2). 18.2%

Table 3. Antibiotic resistance patterns of Salmonella isolated from samples of cattle.

Antibiotic name	Ampicillin	Chloramphenicol	Lincospectin	Nitrofurantoin	Doxyciclin
Resistant percent	36.1%	36.1%	33.3%	8.3%	5.5%

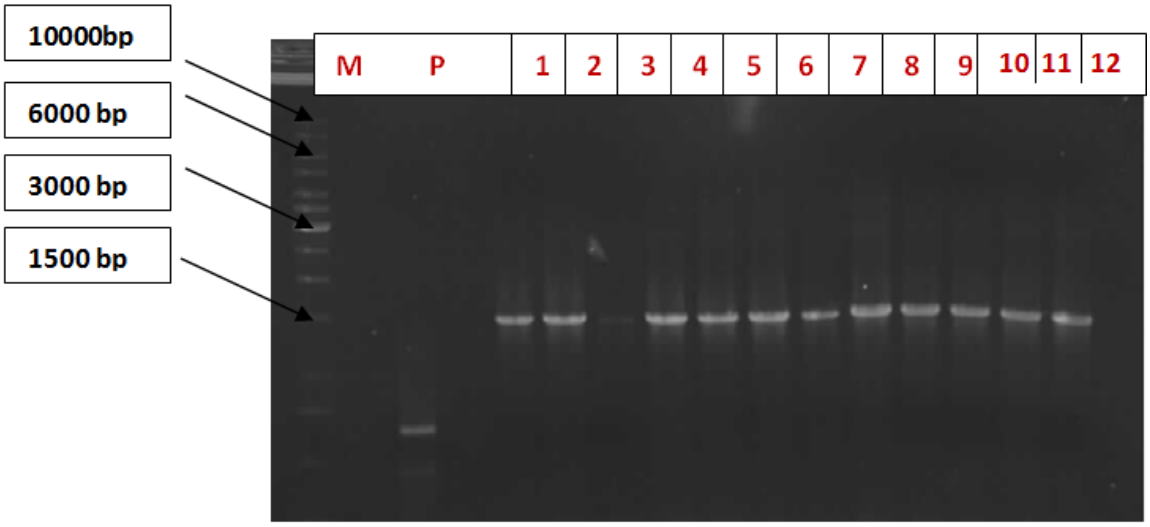

Figure 1. Representative samples determined by PCR and detected by 1 % agarose gel electrophoresis Lane M: 1kb molecular size marker ladder; lane P: positive control, lanes 1 – 12: positive samples.

of *Salmonella typhimurium* was isolated by bacteriological examination of 66 fecal samples collected from calves suffering from watery diarrhea (Riad et al., 1998).

The antibiotic disk diffusion showed that some isolates were resistant to Ampicillin (36.1%), Chloramphenicol (36.1%), Lincospectin (33.3%), Nitrofurantoin (8.3%), and Doxyciclin (5.5%). 33.3% of samples were resistant to three antibiotics. All samples showed the highest sensitivity to Ceftriaxone and Enrofloxacin (Table 3). From sampling in slaughter houses in Uganda in 2010, S. Majalija took only 1.23% of the samples; Salmonella was more resistant to the antibiotics ampicillin, kanamycin, and chloramphenicol that the public health concerns in order to control the use of antibiotics with the present results (Majalija et al., 2010).

The amplified PCR products which were carried out using the universal bacterial *16srRNA* primers and visualized by UV illumination showed the expected bands of about 1500 bp (Figure 1). The results demonstrated a correct genus identification of examined *Salmonella* isolates.

The data shows the results of 36 samples, which were positive by the PCR assay and the results of the same samples was tested using the cultural method for the detection of *Salmonella sp*. More studies on comparative routine microbial cultures and PCR method. The need for the development of rapid and accurate detection methods for *salmonella sp*. has increased in recent years due to the higher incidence of salmonellosis in industrialized countries over the past decades (Tauxe, 1991; Lewis, 1997). Gallegos-Robles et al., (2008) isolated and detected with microbiological and PCR methods, *Salmonella sp.* from fresh beef and cantaloupes. *Salmonella* was detected by the microbiological method in 9 of 20 samples (45%), whereas the pathogen was detected by the PCR in 11 samples (55%). That study demonstrates the utility of the PCR targeting the *invA* gene to determine the presence of *Salmonella sp*. in beef and cantaloupe samples (Gallegos-Robles et al., 2008).

Salmonella strains were detected by direct PCR amplification of the *hilA* gene. The *hilA* primers are specific for *Salmonella* species and the PCR method presented may be suitable for the detection of Salmonella in feces (Pathmanathan et al., 2003).

Assay had been used to detect *Salmonella* in food and beverage samples using suitable primers which were based on specific *invA* gene of Salmonella. The method of PCR demonstrated the specificity of *invA* primers for detection of *Salmonella* as confirmed by biochemical and serological assay. The results of this study revealed that PCR was a rapid and useful tool for detection of *Salmonella* in food and beverage samples (Radji et al., 2010). One searched about detection of *Salmonella sp*. in animal feed samples by PCR after Culture Enrichment. The result of this search showed that 8% of the samples were positive by PCR, compared with 3% with the

conventional method. The reasons for the differences in sensitivity are discussed. Use of this method in the routine analysis of animal feed samples would improve safety in the food chain (Charlotta et al., 2004). *Salmonella* was rapidly detected in dairy cows. All *Salmonella* strains were examined using PCR method. Two oligonucleotide primers were used to detect *Salmonella invA* gene (Eid, 2010). *Salmonella dublin* was detected by PCR amplification of the SopE Gene in Iran (Mirmomeni, 2008). They cause substantial economical loss both directly and and indirectly; directly though mortality and poor growth after clinical disease, and indirectly from animal carriage leading to cases of human *Salmonella* infection which is a serious food-borne infection in man (Ritchie et al., 2001; Donkersgoed et al., 1999; Galland et al., 2000; Rake et al., 2002). The diagnostic method currently in use for *Salmonella enteritis* is a time-consuming and laborious process, that is, culture of the bacteria from the stool samples. Therefore, development of a rapid and sensitive method for the diagnosis of *Salmonella enteritis* is desirable. Several techniques for improving the detection of *Salmonella* serovars in feces, such as the use of a selective culture medium and enzyme-linked immunosorbent assay have been developed. However, problems remain with sensitivity and specificity that have limited routine use of these procedures. PCR technology that allows amplification of a specific fragment of nucleic acid has been used to identify the presence of specific pathogens directly from clinical specimens, such as urine, blood, and cerebrospinal fluid specimens (Cheng-hsun and Jonathan, 1996).

Conventional methods of isolation of *Salmonella* strains take 4–7 days to complete and are therefore laborious and require substantial manpower (Van der Zee et al., 2000). Besides, very small numbers of viable organisms present in the faeces may fail to grow in artificial laboratory media. Molecular testing has been most successful in areas for which conventional micro-biological techniques do not exist, are too slow or are too expensive (Jungkind, 2001). PCR is the best known and most successfully implemented nucleic acid detection technology to date (Nissen et al., 2002).

Conclusion

Salmonella are usually dispersed in the environment and animals are carriers without symptoms of disease. Prevention is not easy and depends on spending on animal husbandry and veterinary. If this patient do not diagnose early and does not treatment of affected animals could be wasting up to 75% of patients. So rapid and exact diagnosis of animal disease can prevented damages inflicted on livestock industry. Thus, there is a need for more reliable and faster methods. The PCR method has proved to be an invaluable tool for this detection.

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