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Detection *Salmonella* spp. Carriers in Camel using Polymerase Chain Reaction and Cultural methods

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ABSTRACT: In this study, polymerase chain reaction (PCR) method and cultivation methods were used and compared to detect carriers of *Salmonella* spp. in camel. 285 feces samples were collected from camels in Kerman province, Iran, in July 2013. Samples were tested for *Salmonella* isolation by culturing techniques and biochemical testes. Feces samples were enriched in enrichment broth and deoxyribonucleic acid (DNA) was extracted and amplified by PCR using specific primers of *Salmonella* invasion gene (*invA*). PCR products were visualized using 1.2% agarose gel electrophoresis. The results of bacterial culturing were negative for all samples, whilst results from the PCR method confirmed the presence of *Salmonella* in 23 camel feces samples (8%). This result indicated that camel may be a reservoir for *Salmonella* spp. Also the PCR method is highly sensitive and rapid for *Salmonella* detection in feces compared to other routine methods.

Keywords: *Salmonella*, *invA* gene, PCR, Camel, Iran

INTRODUCTION

Camel is an important source of milk, wool and meat, as well as transport and handling, must play a larger role than it have in the world. So more scientific attention need to pay on camel researches. *Salmonella* is a worldwide pathogen and can be found in a large number of dairy farms and in many species of animals including mammals, birds, insects, reptiles and human that can result in enterocolitis, septicemia and death (Mirmomeni *et al*., 2008). The genus of *Salmonella* is a gram– negative bacteria in the family of Entrobacteriacea and will be divided into two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonellosis* is an important zoonotic disease (Keusch, 2002). Some adult animals after recover from *Salmonella* infection may become active carriers and excrete the organism in their feces for years (Braun and Methner, 2011). These asymptomatic carrier animals can become a natural reservoir of *Salmonella* responsible for a silent introduction of the bacteria into the food chain and environment, making the control strategies difficult (Maciel *et al*., 2010). Infected animals in farms must be quickly identified and isolated from other ones to prevent and control of spreading of infection.

Therefore, detection of *Salmonella* strains in feces 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 this pathogen (Jadidi *et al*., 2012). Many epidemiological studies on *Salmonella* rely on conventional bacteriological culture methods to detect *Salmonella* in feces samples (Singer *et al*., 2006). The standard technique usually requires a three-step recovery: pre-enrichment, enrichment and selective planting which takes a minimum of 3 days and several additional days for confirmation of presumptive positive results (Kumar *et al., 2005*). Several techniques for improving the detection of *Salmonella* serovars, such as the use of selective culture medium and enzyme-linked immunesorbent assay have been developed. However, because of controversy in interpreting of results and low sensitivity and specificity of these methods, they need to modify and improve those (Zahraei *et al., 2005*). The PCR is a rapid and reliable method for detection and identification of feces samples pathogens. PCR is a molecular biology technique for detection of *Salmonella*, in different kinds of samples, such as meat, milk and feces (Schrank *et al*., 2001; Li and Mustapha, 2002; Santos *et al*., 2003).

Conventional culture methods to detect *Salmonella* spp. are generally labor and time consuming processes, requiring a minimum of 4–6 days (Uyttendaele *et al*., 2003). Several PCR validation studies have reported that the PCR method is one of the most promising of the rapid microbiological methods for the detection and identification of bacteria in a wide variety of samples (Wang and Yeh, 2002). The *invA* gene is the target of many molecular methods because not only it is specific to the *Salmonella* genus, but it is also found in all known pathogen serovars of *Salmonella* spp. (Singer *et al*., 2006). The purposes of this study were to detect the carrier rate in camel in the Kerman region and also to investigate the rapidity and sensitivity of PCR method for identification of *salmonella* and compare it with cultural methods as a conventional strategy.

MATERIALS AND METHODS

A. Sampling

Feces samples were collected with a sterile tongue depressor from 100 camels in July 2013 from Kerman province, Iran. Feces samples were placed into sterile whirl-pak bags, kept at 4°C (Dombek *et al*., 2000).

B. Isolation of Salmonella

Five gr of each sample were inoculated in Selenit F broth (U828504-642, Merck), mixed and incubated at 37°C for 18 h. Each sample enriched in Selenit F was cultured on MacConkey agar (M051, 0500G, Himedia), incubated at 37°C for 24 h and examined for growth. Suspicious bacterial colonies were examined by biochemical testing with the triple sugar iron agar, methyl red Voges–Proskauer reactivity, citrate consumption, urease and decarboxylase activity (Hatta and Smits, 2007).

C. DNA extraction

DNA was extracted as described by reference used with minor modifications (Fadl *et al*., 1995). The modifications were the duration of centrifugation, the amount of enzymes and the addition of cetyltrimethylammonium bromide (CTAB). One ml of the enriched feces samples was transferred to a 1.5 ml micro tube and was centrifuged at 10,000 rpm for 2 min. Pellets were re-suspended in 570 µl of TE (10 mMTris–HCL pH 8, 1 mMNa_2 ethylenediaminetetraacetic acid), 30 µl 10% sodium dodecyl sulphate and 4 µl proteinase K (Fermentas, Germany) in a concentration of 20 mg/ml. Samples in micro-tubes were mixed vigorously before incubation at 37°C in a water bath for 1 h. One hundred ml of 5 M NaCl were added and mixed. One hundred ml of CTAB/NaCl (CTAB 10%, NaCl 0.6 M) were added and mixed. After incubation at 65°C in water bath for 12 min, 500 µl of chloroform/isoamyl alcohol was added and mixed and the samples were centrifuged at 13,000 rpm for 7 min. Five hundred fifty microliters of supernatant were transferred to a fresh micro-tube and equal volume of phenol/chloroform/isoamyl alcohol was added. The samples were mixed and centrifuged at 13,000 rpm for 7 min. The supernatant was transferred to a new micro-tube and 300 µl isopropanol was added to each tube, then centrifuged at 10,000 rpm for 3 min and the pellet was washed with 70% ice-cold ethanol and centrifuged again at 10,000 rpm for 3 min. The final pellet was re-suspended in 50 µl of TE and stored at −20°C until PCR was performed.

D. Oligonucleotide primers

Salmonella-specific primers, S_{139} and S_{141}were based on the DNA sequence of the *invA* gene used to amplify a 284-bp fragment (Freschi *et al*., 2005). The primer sequences are shown in the Table 1.

Table1: Primer sequence and predicted amplified products.

Virulence gene	Oligonucleotide sequence of primers	Amplified product (bp)	Reference
InvA	S_{139}: 5′ GTGAAATTATCGCCGCCACGTTCGAA 3′	284	(Rehn 1992)
	S_{141}: 5′ TCATCGCACCGTCAAAGGAACC 3′		

E. DNA amplification

Amplification reactions were performed in a 25-µl volume containing 12.5 µl Master mix (cinnagen, Iran), 2 µl of R primer, 2 µl of F primer, 2 µl of the template DNA and 6.5 µlof sterile PCR deionized water. Amplifications were performed in CORBETT thermo cycler (Model CP2-003, Australia). Template DNA was initially denatured at 94°C for 5 min, followed by 30 cycles of 45 s denaturation at 94°C, 45 s primer annealing at 58°C, 70 s extension at 72°C and 7 min at 72°C for final extension (Freschi *et al*., 2005). One positive control containing *Salmonella* reference strain ATCC 1730 and one negative control containing water were included in each experiment.

F. Electrophoresis of PCR products

Eight ml of PCR product were mixed with 2 µl of loading buffer for electrophoresis on a 1.2% agarose gel containing ethidium bromide (0.5 µg/ml) for 1 h at 100 V. Visualization was undertaken using of a UV trans illuminator and photographed (Zahraei *et al*., 2005); 1,000 bp DNA ladder (SM0 373) was used as a marker for (PCR) products.

RESULT AND DISCUSSION

The *invA* gene assay was performed in addition to bacteriological culture for each sample. No *Salmonella* colony was obtained using the cultivation methods whilst using the PCR technique, *Salmonella* were present in 8% of samples. So a band with 284 bp weight observed. The presence of 284 bp band in electerophoresis indicates that *invA* gene is present in samples (Fig. 1).

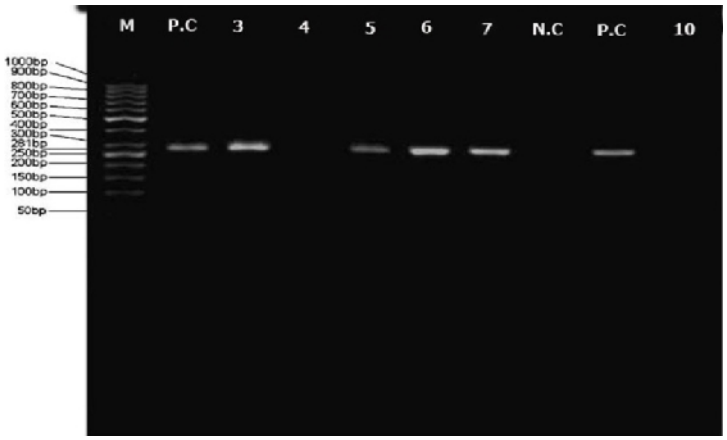

Fig. 1. PCR of camel feces samples for *invA* gene.
M: 1.000 bp marker (Fermentas, Germany).
P.C: *Salmonella* ATCC 1370 (positive control).
N.C: distilled water (negative control).
Lane 3, 5, 6, 7: positive sample.
Lane 4, 10: negative sample.

The goal of this study was to evaluate the suitability of *invA* gene amplification by PCR as a specific method for the detection of *Salmonella* spp. in feces samples and to compare this technique with standard bacterial culture methods for *Salmonella* diagnosis. Furthermore, the carrier rate of *Salmonella* spp. was identified in camel in Kerman province, Iran. One of the critical points of the PCR technique used in this study is the choice of the sequence to be amplified, which must be common to most of the serovars and does not present any homology with other microorganisms. *Salmonella* specific PCR with primers for *invA* gene is specific for the detection of *Salmonella* in many clinical samples (Lambel *et al*., 2000). The method of PCR demonstrated the specificity of *invA* primers for detection of *Salmonella* as confirmed by biochemical and serological assay (Jadidi *et al*., 2012). The *invA* gene codes for protein in the inner membrane of bacteria which is necessary for invasion into epithelial cells and need two oligonucleotide primers to detection (Ahmadi *et al*., 2009). Amplification of an internal fragment of the *invA* gene shown to be essential for *Salmonella* invasion in cell culture (Rahn *et al*., 1992). PCR is an effective, rapid, reliable and sensitive technique for the detection of the *invA* gene of *Salmonella* spp. (Cohen *et al*., 1996). This procedure is very economical and efficient for specific and sensitive detection of *Salmonella* spp. after enrichment of the sample (Myint *et al*., 2006). Of course other primers used as well for detection of *Salmonella* spp. Pathmanathan *et al*. (2003), detected *Salmonella* strains by direct PCR amplification of the *hilA* gene. The results showed that *hilA* primers are specific for *Salmonella* species and the PCR method presented may be suitable for the detection of *Salmonella* in feces. Feces contain a large number of compounds that are inhibitors of PCR. Dead bacteria can also cause false-positive results obtained in the PCR (Jadidi *et al*., 2012). To solve this problem, many researchers before PCR using enriched mediums. Extraction and purification of the gene can reduce the amount of inhibitory substances. Miynt *et al*. (2006), showed that *Salmonella* just after enrichment in specific medium is recognizable by PCR. This modification is in agreement with other researchers and indicates that the inclusion of a pre-enrichment step presents significant advantages over direct extraction of DNA since the enrichment broth is relatively cheap, requires little manipulation, dilutes substances which could inhibit PCR and increases the number of bacterial cells (Oliveira *et al*., 2003). Charlotta *et al*. (2004), showed detection of *Salmonella* spp. in animal feed samples by PCR after culture enrichment. The results of this research 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. Conventional methods of isolation of *Salmonella* strains take 4 to 7 days to complete and are therefore laborious and require substantial manpower (Van der Zee and Huisin't Veld, 2000). Besides, very small numbers of viable organisms present in the feces may fail to grow in artificial laboratory media (Jadidi *et al*., 2012).

Hata *et al*. (2007), showed that 16 of 23 samples of typhoid patients who had negative blood cultures were positive for the presence of bacteria by PCR method. These results show that the PCR with blood sample is a sensitive method for the diagnosis of typhoid fever, and that the PCR with urine and feces could be useful complementary tests. Holger *et al*. (2001), detected of *Salmonella* spp. in the internal organs of pigs that were experimentally infected with the *invA* gene. Wernery (1992), studied two groups of camel in Egypt for detection of *Salmonella* spp. They found that 3% of healthy camels that not show any signs of diarrhea and enteritis were *Salmonella* carriers. Gallegos-Robles *et al*. (2008), isolated *Salmonella* spp. from fresh beef and cantaloupes with microbiological and PCR methods. *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* spp. in beef and cantaloupe samples. The effectiveness of PCR assay to detect *Salmonella* in feces depends not only on the DNA extraction method and the selective enrichment broth used but also on the interaction between them (Ahmadi *et al*., 2009). The extraction and purification of DNA can decrease the amount of inhibitory substances and the selective enrichment can increase the number of viable cells (Freschi *et al*., 2005). The concentration of primers and the annealing conditions also affect the specificity of PCR. High primer concentrations and low annealing temperature allow mis-priming, the products of which will actively compete with the target sequence for primers (Moganedi *et al*., 2007). The molecular-based approach is more rapid for initial detection of *Salmonella* spp. from feces samples (Jadidi *et al*., 2012).Faster identification of *salmonella* in feces would enable earlier implementation of appropriate strategies for treatment, control and prevention (Cohen *et al*., 1996).The results of investigation on the detection of *Salmonella* carriers using the PCR methods in the Urmia, Iran, indicated that carriers of the bacteria in horse, cattle and buffalo were 3,1 and 3 percent, respectively (Ramin *et al*., 2012; Ahmadi *et al*., 2009). According to These results and other studies it seems that rate of *Salmonella* carriers in the camel in Kerman, is like ruminants and equines. So it appears the different hosts are infected from a common source. It seems in Kerman, different hosts including camel can be carriers of *Salmonella*. *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 disease do not diagnose and treat early, could be wasting up to 75% of patients (McQuiston *et al*., 2008). So rapid and exact diagnosis of animal disease can prevented damages inflicted on livestock industry.

PCR offers a great diagnostic tool in comparison to the culture method based on the amount of time required to confirm the presence/absence of *Salmonella*. PCR-based methods with genus-specific primers belonging to *invA*, due to its specificity and sensitivity, are reliable techniques for this proposes.

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