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Detection of *Salmonella* from clinical samples of dogs by PCR*

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ABSTRACT

In present cross sectional study, faecal swabs and blood samples were collected from 250 dogs of Bareilly city, Uttar Pradesh, India and examined for *Salmonella* spp. using standard culture method and polymerase chain reaction (PCR) using primers Cohen-1 and Cohen-2. *Salmonella* were isolated from 1 faecal and 2 blood clots samples. Isolates from faeces were characterized as *S*. Weltevreden [3, 10; r; z_6] and that from blood as *S*. Typhimurium [4, 5, 12; i; 1, 2). Whereas an amplicon of 496 bp was amplified from 6 faecal and 4 blood clot samples. As known positive controls, viz. DNA extract of *S.* Typhi and cell lysate of *S*. Typhimurium, also yielded similar amplicons. The relative sensitivity, relative specificity and accuracy of PCR considering isolation as gold standard was found 100%, 97.17% and 97.20%, respectively. The PCR was found more rapid and sensitive than traditional culture method of isolation from clinical samples which further required biochemical and serological testing for confirmation.

Key words: Dog , PCR, *Salmonella* species

Salmonellosis is one of the most common infectious diseases of human and animals. However, salmonellosis occurs most frequently in latent form in dogs (Kozak *et al*. 2003, Verma *et al.* 2007) and less than 10% infected dogs show acute clinical symptoms of this disease (Green 1998). Chronic asymptomatic carriers often arise from a population infected with *Salmonella* and the difficulty in detecting carriers by culture techniques make them a potential source of environmental contamination (WHO 1988).

Emphasis has already been given to reduce the time required for diagnosis of *Salmonella* infections. The culture and identification of *Salmonella* serovars takes approximately 4 to 7 days (Carter and Chengappa 1991). In addition, *Salmonella* serovars are not detectable in certain clinical samples that contain small numbers of organisms (Fricker 1987). Several techniques for improving the detection of *Salmonella*, such as selective culture methods (Aspinall *et al.* 1992; Thomasen *et al.* 1977), DNA hybridization assays (Curiale *et al.* 1990) and use of immunoglobulin (Rigby 1986, Araj and Chugh 1987), were developed. However, problems with sensitivity and specificity of these methods have hampered their routine application (Garrett *et al.* 1993). Amplification of DNA sequences unique to an organism by PCR improves the speed and sensitivity at which organisms can be detected (Buffone *et al.* 1991). PCR was used for detecting several bacterial species including *Salmonella* from food and clinical samples utilizing specific gene sequences for targeting (Widjojoatmodjo *et al.* 1991, Rahn *et al.* 1992, Cohen *et al.* 1994, Stone *et al.* 1995, Sánchez-Jiménez and Cardona-Castro 2004). The present paper compares the sensitivity, specificity and accuracy of PCR to isolation and characterization methods commonly used in diagnostic laboratories.

MATERIALS AND METHODS

Bacterial isolation: Attempt was made to isolate and characterize *Salmonella* from the faeces and blood collected from 250 apparently healthy dogs. Faecal samples were obtained by swabbing the rectum with sterile cotton-wool swabs, which were immediately transferred to the labelled sterile test tubes and brought on ice to the laboratory. Each of the 250 faecal samples were transferred for pre-enrichment to 10.0 ml of buffered peptone water (BPW) and incubated at 37°C for 15–18 h. After pre-enrichment, 0.1 and 1.0 ml of aliquot from BPW was transferred to 10.0 ml of Rappaport Vassidalis broth (RV) and 10.0 ml of tetrathionate broth (TTB) and incubated at 37°C for 24 h for enrichment.

About 3.0 ml blood collected aseptically from 250 dogs

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by using labelled sterile disposable syringes. The clotted blood was transported to laboratory on ice. The blood clots were transferred to 50.0 ml of bile broth and incubated at 37°C for 48–72 h for pre-enrichment. The enrichment was done as described for faecal sample.

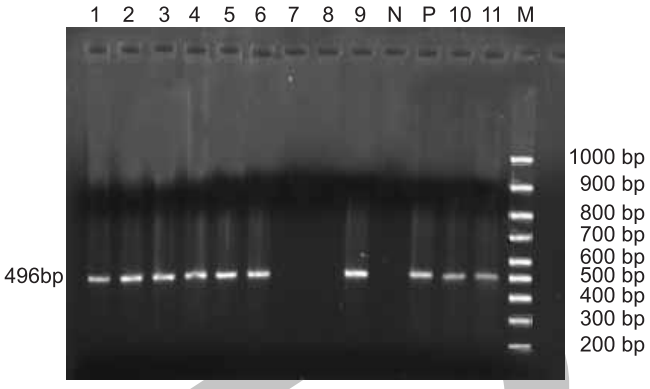
Thereafter, a loopful of inoculum from the enrichment broths was streaked on to Hektoen enteric agar (HEA) plates and incubated at 37ºC for 24 h. Presumptive *Salmonella* colonies which appeared as smooth, transparent, with or without black centre having greenish periphery, were picked up and characterized biochemically (Edwards and Ewing 1972) before submitting for serotyping at National *Salmonella* Centre (Veterinary), Indian Veterinary Research Institute, Izatnagar.

Polymerase chain reaction (PCR): After 24 h of selective enrichment in TTB, 1.0 ml aliquot was centrifuged at 8000 rpm for 10 min, to collect the pellet. All the media contents were drained off by gentle tapping. The pellet was resuspended in 100µl of sterile distilled water, boiled for 10 min and immediately transferred onto ice (snap chilling). It was stored at–20ºC till further use. Just before use, the bacterial lysate was mixed properly and centrifuged at 4000 rpm for 2 min to settle the cell debris and an aliquot of 5µl of the supernatant was used as template for PCR assay. To determine *Salmonella* in faeces and blood clots, 496 bp fragment of a conserved sequence of the *his* J gene was amplified (Cohen *et al.* 1994) by PCR, using primer 1 (5' ACT GGC GTT ATC CCT TTC TCT GGT G) and 2 (5' ATC TTG TCC TGC CCC TGG TAA GAG A) custom synthesized and template DNA. Reaction mix (25µl) consisted 5µl of the template, 2.5µl of 10 × Taq Polymerase reaction buffer, 1.5 mM $MgCl_2$, 300µM dNTP mix, 37.5 pM of each of the 2 primers and 1 Unit of Taq DNA polymerase. For amplification in thermocycler an initial denaturation (5 min at 94ºC) was followed by 30 cycles of denaturation (1 min at 94ºC), annealing (1 min at 55ºC) and extension (90 sec at 72ºC) and a step of final extension for 10 min at 72ºC. The amplicon of 496bp was visualized under UV illuminator after agarose gel electrophoresis (5 volts/cm) using 1.5% agarose made in 0.5X Tris-borate buffer (TBE) containing ethidium bromide (0.5µg ml^{-1}). Known positive control, viz. DNA extract of *S.* Typhi and cell lysate of *S.* Typhimurium were also placed along with samples.

Relative sensitivity and specificity: The relative sensitivity, specificity and accuracy of PCR for detection of *Salmonella* spp. was evaluated following the method of Mc Diarmid and Hellstrom (1987) in comparison to the traditional culture method of isolation.

RESULTS AND DISCUSSION

The faecal and blood samples collected from 250 apparently healthy dogs, *Salmonella* was isolated from only 1 faeces (0.4%) and 2 blood clots (0.8%) samples. Isolates from faeces were characterized as *S.* Weltevreden [3, 10; r; z_6] and that from blood as *S.* Typhimurium [4, 5, 12; i; 1, 2), whereas an amplicon of 496 bp was amplified from 6 faecal (2.4%) and 4 (1.6%) blood clot samples. As known positive controls, viz. DNA extract of *S. Typhi* and cell lysate of *S. Typhimurium*, also yielded similar amplicons (Fig.1). Interestingly, none of the faecal and blood samples positive either by isolation or PCR technique was from same dog.



Lane M: 100 bp molecular weight marker; Lane P: Positive control; Lane N: Negative control; Lane 1-11: Cell lysate (faecal and blood samples)

Fig 1. PCR assay for detection of *Salmonella* in dogs

Comparison of isolation and PCR results revealed that all the 3 dogs positive for *Salmonella* spp. by culture were positive with PCR too. However, 7 (5 faecal and 2 blood samples) more dogs were found positive by PCR. The relative sensitivity, specificity and accuracy of PCR, considering isolation as gold standard, was calculated to be 100%, 97.17% and 97.20% respectively.

In present cross-sectional study, from 250 dogs from which faecal swabs as well as blood samples were collected. *Salmonella* were isolated from 1 faecal swab and 2 blood clots. Isolates were characterized *S.* Typhimurium from blood and *S.* Weltevreden from faeces. The occurrence of *S.* Typhimurium in dogs is common and reported by many workers in India (Verma and Gupta 1989, Meenu 2002) and abroad (Kallo and Hasso 2001, Fukata *et al.* 2002). Faeces are the sample of choice for isolation of majority of serovars of *Salmonella*, as intestine is the seat of predilection. However, in the present study the *S.* Typhimurium were isolated from blood clots. It may be due to colonization of the pathogen in the intestinal lymph nodes after invasion from where it gains entry into the blood stream leading to systemic infection. Hence for *S.* Typhimurium blood clots may be another good choice as observed earlier (Khan 1970, Fukata *et al.* 2002). *S.* Weltevreden, though common in many foods of animal origin and has been regarded as the emerging serovar all over the globe (www. *Salmonella*.org, www.up.gov.up.nic.in/ivri/nsc) has rarely been reported from dogs. Faecal excretion of this zoonotic serovar of *S.* Enterica by apparently healthy

dogs is of public health significance.

The overall prevalence of *salmonellosis* as determined by PCR was 4.0% indicating it as more sensitive than isolation technique (Amavisit *et al*. 2001, Claudia *et al.* 2002, Babu, 2003, Jyoti 2004). Several factors interfere with the isolation of *Salmonella* spp. from clinical specimen, viz. the contaminating organisms present in specimen inhibit *Salmonella* isolation, antibiotics therapy to infected animals can retard the multiplication of *Salmonella* organisms resulting in their periodical shedding only and that too in very low numbers, particularly in carriers (WHO 1988). The higher sensitivity of PCR may be because of its ability to detect very small number of viable as well as the DNA of non-viable *Salmonella* (Cohen *et al.* 1994, 1996). Better performance of PCR may also be due to the protocol used in the current study, viz. pre-enrichment with buffered peptone water followed by enrichment with TTB which might help in resuscitating the injured organism (Chiu and Ou 1996, Gouws *et al.* 1998, Soumet *et al.* 1999, Carli *et al.* 2001, Feder *et al.* 2001). Stone *et al.* (1995) observed in their study that TTB were inhibitory to PCR but in the present study as well as the earlier studies of our laboratory (Babu 2003, Jyoti 2004) and by others (Carli *et al*. 2001) also no such inhibition was observed. This may be due to suspension of pellet in sterile distilled water resulting in the dilution of the inhibitory action of the detrimental ingredient(s) in the medium. The choice of primer set is also crucial for the sensitivity of PCR assay as it is evident from the study of Claudia *et al.* (2002) that the PCR assay using invE-A primer had a sensitivity of 80% as compared to 93.3% using *his* J primers. The later was used in present study.

The isolation of *Salmonella* by culture method is laborious, time consuming and takes at least one week time (Fricker 1987). However, the use of PCR has increased the rapidity and sensitivity of diagnosing infectious diseases including salmonellosis (Carli *et al*. 2001). The presence of low level of *Salmonella* organisms are common in samples from animals suspected of having salmonellosis with no clinical signs and negative to culture technique. In such cases, PCR could be applied to identify the etiological agent. Similar to Ward *et al.* (2005) findings, in present study too all culture-positive samples were PCR-positive and 5 faecal (2.0%) and 2 blood clots (0.8%) samples negative to isolation method were positive to *Salmonella* in PCR. The findings indicate PCR assay as a simple, rapid, sensitive, reliable and reproducible method for the identification of *Salmonella* that will aid in surveillance, prevention and control of this pathogen as reported by Perera and Murray (2008) also.

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REFERENCES

Amavisit P, Browning F G, Lightfoot D, Church S, Anderson G A, Whithear K G and Markham P F. 2001. Rapid PCR detection of *Salmonella* in horse faecal samples. *Veterinary Microbiology* **79**: 63–74.

Araj G F and Chugh T D. 1987. Detection of *Salmonella* spp. in clinical specimens by capture enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology* **25**: 2150–53.

Aspinall S T, Hindle M A and Hutchinson D N. 1992. Improved isolation of *Salmonella*e from faeces using a semisolid Rappaport Vassiliadis medium. *European Journal of Clinical Microbiology and Infectious Disease* **11**: 936–39.

Babu N. 2003. 'Epidemiological studies on *Salmonella* infection in horses.' M.V.Sc. thesis submitted to Deemed University, IVRI, Izatnagar, India

Buffone G J, Demmler G J, Schimbor C M and Greer J. 1991. Improved amplification of cytomegalovirus DNA from urine after purification of DNA by glass beads. *Clinical Chemistry* **37**: 1945–49.

Carli T K, Unal B C, Caner V and Eyigor A. 2001. Detection of *Salmonella*e in chicken faeces by a combination tetrathionate broth enrichment, Capillary PCR and Capillary Gel Electrophoresis. *Journal of Clinical Microbiology* **39** (5): 1871–76.

Carter, G R and Chengappa M M. 1991. Enterobacteriaceae. *Essentials of Veterinary Bacteriology and Mycology.* 4th edn, Pp. 150–64. Lea and Febiger, Philadelphia.

Chiu C H and Ou J J. 1996. Rapid identification of *Salmonella* serovars in faeces by specific detection of virulence genes inVA and spvC by an enrichment broth culture- multiplex PCR combination assay. *Journal of Clinical Microbiology* **34**: 2619–22.

Claudia GW, Hutcheson H J, Kim L M, Bolte D, Dargartz J T, Marley P, Power B and Jessen M. 2002. Identification of two phylogenetically related organisms from faeces by PCR for detection of *Salmonella* spp. *Journal of Clinical Microbiology* **40 (**4): 1487.

Cohen N D, Wallis D E, Neibergs H L, Hargis B M, Simpson R B and Mc Gruder E B. 1994. Genus specific detection of *Salmonella*e in equine species by use of polymerase chain reaction. *American Journal of Veterinary Research* **55** (8): 1049–54.

Cohen N D, Martin L J, Simpson R B, Wallis D E and Neibergs H L 1996. Comparison of polymerase chain reaction and microbiological culture for detection of *Salmonella*e in equine species and environmental samples. *American Journal of Veterinary Research* **57 (**6): 780–86.

Curiale M S, Klatt M J and Bartlett C L. 1990. Colorimetric deoxyribonucleic acid hybridization assay for rapid screening of *Salmonella* in foods. *Journal of Associations of Analytical Chemistry* **73**: 248–56.

Edwards P R and Ewing W H. 1972. *Identification of Enterobacteriaceae.* 4th edn. Elsevier Science, Burgees Pub Co, USA.

Feder I, Jerome C N, John G, Teresa Y, Jan M S, Richard O, Mark L T and John B L. 2001. Comparison of cultivation and PCR-hybridization for detection of *Salmonella* in porcine faecal and water samples. *Journal of Clinical Microbiology* **39** (7): 2477–84.

Fricker C R. 1987. The isolation of *Salmonella*s and campylobacters. *Journal of Applied Bacteriology* **63**: 99–116.

Fukata T, Naito F, Yoshida N, Yamaguchi T, Mizumura Y and Hirai K. 2002. Incidence of *Salmonella* infection in healthy dogs in Gifu Prefecture, Japan. *Journal of Veterinary Medical Science* **64(**11): 1079–80.

Garrett C T, Ferreira-Centeno A and Nasim S. 1993. Molecular diagnostics: issues of utilization, regulation, and organization. *Clinical Chemistry Acta* **217**: 85–103.

Gouws P A, Vesser M and Brozel V S. 1998. A polymerase chain reaction procedure for the detection of *Salmonella* spp within 24 hours. *Journal of Food Protection* **61**: 1039–42.

Green E C. 1998. *Infectious Diseases of Dogs*. Pp 235–40. W B Saunders Co., Philadelphia.

Jyoti J. 2004. 'Studies on epidemiology of salmonellosis in equines.' M.V.Sc. thesis submitted to Deemed University, IVRI, Izatnagar, India.

Kallo O J and Hasso S A. 2001. Prevalence of *Salmonella* serotypes in dogs and their sensitivity to antimicrobials. *Iraquian Journal of Veterinary Science* **14**1: 67–71.

Khan A Q. 1970. Salmonella infection in dog and cat in Sudan. *Brajelian Veterinary Journal* **126**: 607.

Kozak M, Horosova K, Lasanda V, Bilek J and Kyselova J. 2003. Do dogs and cats present a risk of transmission of salmonellosis to humans? *Bratisl Lek Listy* **104**: 323–28.

Mc Diarmid S C and Hellstrom J S. 1987. An intradermal test for the diagnosis of brucellosis in extensively managed cattle herds. *Preventive Veterinary Medicine* **4**: 361.

Meenu 2002. 'Study of heterogeneity in Indian isolates of *Salmonella* paratyphi B.' M.V.Sc. thesis submitted to Deemed University, IVRI, Izatnagar, Uttar Pradesh, India.

Perera K and Murray A. 2008. Development of a PCR assay for the identification of *Salmonella* Enterica serovar Brandenburg. *Indian Journal of Medical Microbiology* **57**: 1223–27.

Rahn K, De Grandis S A, Clarke R C, McEven S A, Galan J E, Ginocchio C, Curtiss III R and Gyles C L. 1992. Amplification of an invA gene sequence of *Salmonella* Typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella. Molecular and Cellular Probes* **6**: 271–79.

Rigby C E. 1984. Enzyme-linked immunosorbent assay for the detection of *Salmonella* lipopolysaccharide in poultry specimens. *Applied Environmental Microbiology* **14**: 145–52.

Sánchez-Jiménez M M and Cardona-Castro N. 2004. Validation of a PCR for diagnosis of typhoid fever and salmonellosis by amplification of the hilA gene in clinical samples from Colombian patients. *Journal of Medical Microbiology* **53**: 875–78.

Soumet C, Ermel G, Rose V, Rose N, Droin P, Salvat G and Colin P. 1999. Identification by a multiplex PCR based assay of *Salmonella* Typhimurium and *Salmonella* Entritidis strains from environmental swabs of poultry houses. *Letters in Applied Microbiology* **29**: 116.

Stone G G, Oberst R D, Hays M P, McVey S, Gallnd J C, Curtiss III R, Kelley S M and Chengappa M M. 1995. Detection of *Salmonella* Typhimurium from rectal swabs of experimentally infected Beagles by short cultivation and PCR hybridization. *Journal of Clinical Microbiology* **33 (**5): 1292–95.

Thomasen B M, Dodd D J and Cherry W B. 1977. Increased recovery of *Salmonella*e from environmental samples enriched with buffered peptone water. *Applied Environmental Microbiology* **34**: 270–73.

Verma A K, Sinha D K and Singh B R. 2007. Salmonellosis in apparently healthy dogs. *Journal of Veterinary Public Health* **5**1: 37–39.

Verma J C and Gupta B R. 1989. Occurrence of *Salmonella* serotypes in animals in India III. *Indian Journal of Compendium Microbiological, Immunological and Infectious Disease* **10**: 1.

Widjojoatmodjo M N, Fluit R, Torensma G P, Verdonk G P H T and Verhoef J. 1991. The magnetic immuno pol;ymerase chain reaction assay for detection of *Salmonella*e in fecal samples. *Journal of Clinical Microbiology* **30**: 3195–99.

Ward M P, Alinovi C A, Couëtil LL and Wu C C. 2005. Evaluation of a PCR to detect Salmonella in fecal samples of horses admitted to a veterinary teaching hospital. *Journal Veterniary Diagnostic Investigation* **17(**2): 118–23.

World Health Organization. 1988. Salmonellosis control: the role of animal and product hygiene. *WHO Technical Report* Series No. 774, W H O, Geneva.