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Salmonella enterica Subsp. *Enterica* Serovar Reading Infection in Dairy Cattle and Buffaloes Suffering from Chronic Diarrhea

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ABSTRACT

The study was aimed at dairy cattle and buffaloes presented with the history of chronic diarrhea to isolate and detect *Salmonella* spp. Fecal samples were collected directly from the rectum of diarrheic cattle. Isolation and identification of the microorganisms was performed and confirmed on the basis of their morphology, staining, cultural, biochemical tests and sero-typing. *Salmonella* positive animals presented variable degree and frequency of diarrhea. Salmonellosis was confirmed in seven per cent of cattle and buffaloes suffering from chronic diarrhea. The serotype detected in the 7 *Salmonella* strains was *Salmonella enterica* subsp. *enterica* serovar Reading. *Salmonella* isolates recovered from dairy cattle had relatively variable resistance to various antimicrobial agents. The antimicrobial susceptibility pattern of the isolates showed isolates were resistant to amoxicillin, tetracycline, ampicillin, ceftriaxone. Whereas 87.5 percent, 75.0 percent and 62.5 percent susceptible to ciprofloxacin, gentamicin and co-trimoxazole respectively. This study provides updated information on the bio-incidence and susceptibility patterns of *Salmonella* in dairy animals suffering chronic diarrhea. Isolation and serotyping of *Salmonella enterica* subsp. *enterica* serovar Reading is new to the study and contribute to our understanding that there is shift in increases in susceptibility of dairy herds to acquire new strains.

Key words: Antimicrobials, Chronic diarrhea, Dairy cattle and buffaloes, PCR, Salmonella.

INTRODUCTION

Chronic diarrhea is one of the costly pathologic conditions affecting livestock (Wyatt *et al.* 2010; Radostits *et al*. 2000). Diagnosis and therapy of chronic diarrhea is very challenging for clinicians (Davison *et al*. 2005; Merritt 1994). Clinical experience and feedback of field practitioners show that chronic diarrhea in dairy animals is frequently encountered. It has significant impact on economic returns owing to its affects on animals general health status, longevity in the herd, losses through premature culling, weight loss, reduced milk production, increased treatment costs and the possibility of the spread of infection to healthy herd mates (Raizman *et al.* 2007). Salmonellosis is one of the confirmed bacterial causes of diarrhea in all age groups of dairy cattle and buffalo (Wray *et al*. 2004). Outbreaks of disease are costly for producers because of increased mortality and treatment costs (Smith 1996). There are few serotypes that are associated with cattle however during last few decades, it has been seen that there is change in shift of prevalence of strains and serotypes associated with dairy animals (Smith 1996; Gossner *et al.* 2016). Although several studies have examined the prevalence of faecal shedding of *Salmonella* in dairy farms very little information is available regarding the occurrence of chronic diarrhea associated with *Salmonella* infections in adult cattle (Mcevoy *et al*. 2003; Wells *et al*. 2001). Efficient laboratory methods for isolation, identification and typing of Salmonella are essential elements in Salmonella control programs (Huston 2002). The ideal method should have a high sensitivity and specificity and at the same time should be simple, rapid and inexpensive.

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During the last decade, there has been an alarming increase in the appearance of antibiotic-resistant *Salmonella* that may be associated with the irrational use of antimicrobial agents in dairy animals (Blau 2005; Tadesse *et al*. 2017). It is possible that indiscriminate use of antimicrobial use at the farm level increases the susceptibility of dairy herds to acquire new strains (Verma *et al*. 2005). In order to find out exact etiology of chronic diarrhea, where it can be inflammatory in nature, isolation and detection of *Salmonella* and the antibiogram pattern is of paramount importance in order to design methods for minimizing its possible transmission in the herd. Therefore, detection of *Salmonella* spp in fecal samples is not only important for the establishing the etiology of chronic diarrhea in dairy animals but can help to eliminate the disease at the farm level. The purpose of this study was to utilize molecular technique along with

traditional isolation method in order to detect *Salmonella* as one of the causes of chronic diarrhea in dairy cattle.

MATERIALS AND METHODS

Study was conducted from March 2013 to March 2015. Fecal samples were collected from 102 dairy cattle with history of persistent or intermittent chronic diarrhea. Detailed history was recorded with respect to age, epidemiology, feeding pattern, body condition, duration, frequency and nature of diarrhea. Comprehensive clinical examination including rectal examination for determining abdominal abnormalities was carried out to determine the nature and severity of diarrhea. Fecal samples were collected aseptically directly from the rectum and processed within 4-8 hours for the isolation and identification of *Salmonella*. Fecal smears were found negative for ova/cyst and fecal microscopy was done to rule out Acid Fast Bacilli (AFB). Physical and clinical examination was routinely done and all the vital parameters were within the normal ranges.

Isolation of *Salmonella*

Approximately 1 gram of feces was directly inoculated in 9ml of buffered peptone water and another 0.1 ml portion was transferred to10 ml of Rappaport Vassilidis (RV) broth, incubated overnight at 42°C (HiMedia, Mumbi). From the selective enrichment, samples were inoculated on Hekton enteric agar (HE) and incubated at 37°C overnight (18-24 h). Finally for selective isolation suspected colonies from the HE agar plate were inoculated on Brilliant Green Agar plates (HiMedia, Mumbi). Typical colonies were subjected to biochemical tests using triple sugar iron (TSI), methyl Red, voges-Proskauer (MRVP), urea, lysine iron agar, indole tests (Himedia, Mumbi) and identified using standard methods (Sambrook *et al*. 2000; Piddock *et al* 2002).

Isolates presumed to be *Salmonella* were serogrouped using serogroup-specific sera and sent to the National Escherichia and Salmonella centre Kasoli (HP) for serotyping.

PCR was also performed and chromosomal DNA extraction of DNA was performed according to the standard method given with slight modification (Cohen *et al.* 2002).

Primers

Two universal oligonucleotides primers were obtained from ITC (India). The following primer pair was used in PCR reactions to obtain a 496 bp product: 5' ACT GGC GTT ATC CCT TTC TCT GGT G 3' and 5' ATG TTGTCC TGC CCC TGG TAA GAG A 3'. PCR was performed in 25 µL reaction volumes; a PCR mixture was prepared consisting of forward and reverse primer (1.0 µM each), Taq Master mix 12.5 µL and 5 µL of sample. The reaction was completed upto 25 µL with nuclease free water. A positive and negative control containing the template DNA from *Salmonella* spp was also included in the experiment. The reaction was carried out for 30 cycles and PCR was programmed to 1 min for denaturation at 94°C, annealing at 55°C for 1 min and extraction at 72°C for 1.30 min, concluded with a 10 min extension phase at 72°C and stored at 20°C (Eid 2010; Gouws *et al.*1998). The PCR products obtained using Salmonella species-specific primers were electrophoresed on 1% agarose gels, stained with ethidium bromide and visualized under utraviolet light in a gel doc system (Biorad, USA).

Antimicrobial susceptibility test

The antimicrobial susceptibility test was performed using Kibry-Bauer disk diffusion method on Muller Hinton agar (HiMedia-India). Isolates were tested for the following antibiotics; ciprofloxacin (5 µg), chloramphenicol (30 µg), tetracycline (30 µg), ampicillin (10 µg), streptomycin (10 µg), enrofloxacin (30 µg), co-trimoxazole (Thrimethoprim-sulfmethoxazole) (25 µg), gentamycin (10 µg), amoxicillin (10 µg) and ceftriaxone (30 µg) (HiMedia- India). According to the width of the inhibitory zone, the pattern of drug sensitivity was determined as susceptible and resistant.

RESULTS AND DISCUSSION

Salmonellosis was confirmed in seven per cent of cattle and buffaloes suffering from chronic diarrhea by faecal culture and PCR. Moderate weight loss was observed in 28.5 per cent (2/7) of animals and 71.4 per cent (5/7) of animals had foul smelling Faecal odor. Mucoid feces were seen in 57.1 per cent (4/7) whereas blood tinged mucoid feces were found in one of the *Salmonella* infected animal (Table 1). Among *Salmonella* infected adult dairy cattle, clinical disease (diarrhea) can range in animals from 0 per cent to nearly 100 per cent (Genovese *et al*. 2004). Diarrhea was reported in 91 per cent of farms that experienced clinical salmonellosis in animals at the time of diagnosis (Van Kessel *et al*. 2007). It is also reported diarrhea and depression as

Table 1: Clinical findings in chronic Salmonellosis in dairy animals.

Animal	Duration of Diarrhea	Course of diarrhea	Consistency of faeces	Abnormal constituents in faeces	Odor of faeces
1.	1-month	Persistent	Watery	Mucus	Foul smelling
2.	1-1/2 month	Intermittent	Loose	Mucoid feces/ Blood	Foul smelling
3.	25 days	Persistent	Loose	Mucus/ shreds	Normal
4.	1-month	Persistent	Watery	Mucoid feces	Foul smelling
5.	3-months	Intermittent	Loose	Mucus	Foul smelling
6.	2-1/2 month	Intermittent	Loose	-	Foul smelling
7.	4-months	Intermittent	Loose	-	Normal

the most frequently observed clinical sign in adult dairy cattle due to Salmonellosis (Pasmans *et al*. 2007). Blood in faeces in animals was reported in seven farm animals with diarrhea as a clinical sign. Clinical infection of adult cattle with salmonellosis was associated with diarrhea with or without dysentery (Warnick *et al.* 2003).

Initially fecal microscopy was done to rule out presence of (cyst or ova) parasitic infection. All the positive samples were acid fast bacillus (AFB) negative by Zeihl Neelsen staining technique that rules out concurrent paratuberculosis infection. All isolates yielded negative results for Vogues-Proskauer (VP) test, urease, indole production and lactose fermentation. These isolates were positive for methyl red (MR), triple sugar iron agar (TSI) test and glucose fermentation with gas production. These reactions confirmed *Salmonella* spp. On Gram's staining, *Salmonella* isolates were identified as short single gram negative rods. The data confirmed the results of seven samples, which were positive by the PCR assay and the results of the same samples were positive by the cultural method for the detection of *Salmonella* spp. The amplified PCR products using oligonucleotide primers, visualized by UV illumination showed the expected bands of about 496 bp (Fig 1). The results demonstrated a correct genus identification of examined *Salmonella* isolates and seven samples were PCR positive for *Salmonella sp*. The serotype detected in the 7 *Salmonella* strains was *Salmonella enterica* subsp*. enterica* serovar Reading. Simultaneously faeces from healthy cattle were culture negative and PCR negative for *Salmonella.* Genovese (1994) also reported rapid detection of *Salmonella* from the diarrheic faeces of dairy cows by PCR method using two oligonucleotide primers. No other serotypes were identified in all *Salmonella* isolates. The utility of PCR to determine the presence of *Salmonella* spp. in beef cattle was demonstrated (Velinga *et al.* 2002). He isolated 9 *Salmonella* spp from 20 samples, in contrast to the pathogen detected in 11 samples (55%) by PCR. Out of 1124 faecal samples, 34 were detected by PCR technique by biochemical and molecular assay to detect and diagnose *Salmonella* in cattle. *Salmonella* was in 10.7 percent (21/195) of diarrheic faecal samples (Jones *et al*. 2004). It was observed in a study that higher prevalence of salmonellosis among adult dairy cattle of about 27 per cent (Jadidi *et al*. 2012). *Salmonella enterica* serovar *Typhimurium* and *Dublin* were earlier mainly isolated from cattle with the isolation rate decreasing year by year (Addis *et al*. 2011). With the isolation of *Salmonella enterica* serovar Reading, from dairy animals the changing pattern of *Salmonella* serotypes can be confirmed. The need for the development of rapid and accurate detection methods for *Salmonella* has increased in recent years due to higher incidence of the disease (Akoachere *et al*. 2009).

Antibiogram of *Salmonella* isolates

The *Salmonella enterica* subsp*. enterica* serovar Reading showed highest sensitivity to enrofloxacin and ciprofloxacin (87%) followed by gentamicin (75%) and tetracycline (66.7%). *Salmonella* isolates showed moderate sensitivity to co-trimoxazole (62.5%), ceftriaxone and chloramphenicol (59.7%). Isolates were resistant to streptomycin (13%), ampicillin and amoxicillin (100%). Effectiveness of enrofloxacin and ciprofloxacin against maximum of the *Salmonella* isolates suggested that enrofloxacin was still drug of choice for control of salmonellosis. Similar to the present findings, variable efficacy of tetracycline, ceftriaxone, co-trimoxazole and chloramphenicol were recorded against majority of the isolates of *Salmonella* (Smith 2014). However these drugs recorded low sensitivity against *Salmonella* isolates from other domestic animals indicating that the drug resistance pattern in *Salmonella* is under stage of flux. Complete resistance of *Salmonella* isolates against two or more of antimicrobials (100%) (amoxicillin and ampicillin) has been recorded (Varma *et al*. 2005). This finding is in line with previous reports from tropical and sub-tropical areas (Khaitsa *et al*. 2007). The antimicrobial resistance may be due to the emergence of drug resistant strains following the extensive and indiscriminate use of antimicrobial agents for therapy as reported by (Suresh *et al*. 2006). Tetracycline

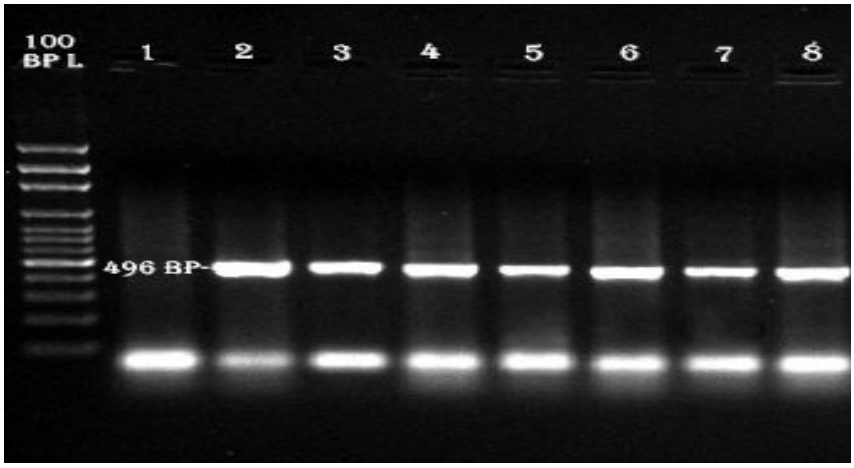

Fig 1: Positive samples determined by PCR and detected by 1% agarose gel electrophoresis. Lane ladder: 100 bp molecular size marker ladder; lanes 2nd-8th: positive samples.

and chloramphenicol resistance was variable among current isolates and very low to very high rates of resistance to these therapeutic agents have been reported (Pathmanathan 2003; Alexander *et al*. 2009). Antimicrobial agents are used as therapy, prophylaxis and as growth promoters in dairy farming. All of these are responsible for promoting selective pressure on bacterial populations and thereby antibiotic resistances are developed (Haque *et al*. 1998). Ciprofloxacin, gentamicin, co-trimoxazole and enrofloxacin showed good antimicrobial activity against *Salmonella* isolates and were considered to be very good alternatives for clinical treatment. Such results have been reported earlier as well and are comparable with the results of this study (Callaway *et al*. 2005; Hghi *et al*. 2009). In the study the positive cases were sporadic in occurrence without any history of previous outbreak. The fact may be that illness is not due to recently acquired infection, but to activation by some other stress factor of a latent infection. Dairy animals with chronic diarrhea act as carrier and a source of infection for other herd animals. The occurrence of *Salmonella* spp from the faeces of diarrheic cattle is a concern to the dairy farms, since they are potential source of infections and contamination can cross from one species to another (Kalambhe *et al.* 2016). Conventional methods of isolation of *Salmonella* strains is time consuming and take 4-7 days to complete, therefore, development of a rapid and sensitive method for the diagnosis of *Salmonella* spp is desirable (Lewis 1997; Van *et al*. 2000 Hare *et al*. 2003).

Salmonella positive animals presented with loose to profuse watery diarrhea. Salmonellosis was diagnosed in 6.9 per cent of cattle and buffaloes suffering from chronic diarrhea by faecal culture and PCR. The serotype detected in the 7 *Salmonella* strains was *Salmonella enterica* subsp. *enterica* serovar Reading. *Salmonella* isolates showed highest sensitivity to enrofloxacin (87%), ciprofloxacin (87%), gentamicin (75%) and tetracycline (66.7%) and all isolates showed resistance to ampicillin and amoxicillin.

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Conflict of Interest Statement

All authors acknowledge and declare there is no conflict of interest.

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