PREVALENCE OF *CRYPTOSPORIDIUM* SPP. INFECTION IN CATTLE AND BUFFALO CALVES IN AND AROUND MUMBAI REGION

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BY

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Enrolment No : V/06/0105

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PGR - ANNEXURE – XIII

DECLARATION BY THE STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled, **"PREVALENCE OF *CRYPTOSPORIDIUM* SPP. INFECTION IN CATTLE AND BUFFALO CALVES IN AND AROUND MUMBAI REGION"** or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

Date : / / 2013

Signature

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PGR - ANNEXURE – XV

CERTIFICATE

This is to certify that the thesis entitled, **"PREVALENCE OF *CRYPTOSPORIDIUM* SPP. INFECTION IN CATTLE AND BUFFALO CALVES IN AND AROUND MUMBAI REGION"** submitted by **MISS HINGOLE ARATI CHANDRAMUNI** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **MASTER OF VETERINARY SCIENCE (M.V.Sc)** in Veterinary Parasitology has been approved by the student's Advisory Committee after the examination in collaboration with the External Examiner.

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Mumbai.

Arati Chandramuni Hingole

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LIST OF ABBREVIATIONS

%	: Per cent
µl	: Microliter
^{0}C	: Degree Celsius
µg	: Microgram (s)
µm	: Micrometer
AGE	: Agarose gel electrophoresis
BLAST	: Basic local alignment search tool
bp	: Base pair
DNA	:Deoxyribonucleic acid
DW	: Distilled water
dNTPs	: Deoxynucleotide triphosphates
et al.	*: et alii*
gm	: Gram
h	: Hour
I.U.	: International unit
mg	: Milligram

ml	: Millilitre
mM	: Millimolar
NCBI	: National Center for Biotechnology Information
OD	: Optical density
PCR	: Polymerase chain reaction
pg	: Picogram
pmole	: Picomole (s)
RNA	: Ribonucleic acid
rpm	: Rotation per minute
TE	: Tris-EDTA Buffer
sec	: Seconds
Taq	*: Thermus aquaticus*
TAE	: Tris acetate EDTA buffer
U	: Unit
UV	: Ultra violet
V	: Volts
viz	: Namely
v/v	: Volume by volume
w/v	: Weight by volume

1. INTRODUCTION

Characteristic of agrarian economy, animal husbandry goes hand in hand with agriculture in India. A predominant aspect of animal husbandry in India is domestication of cattle and buffaloes, the history of which dates back to the Vedic times. The popularity of this practice does not come as a surprise when one takes into consideration the way of life in rural India. The onus of agricultural operations like ploughing, threshing and transport of agricultural produce is shouldered by cattle and buffalo bulls even today, despite the advantages provided by mechanization. Milk that is obtained from these animals is central to the diet of many people. It is also one of the few sources of protein for a significant vegetarian population of India. The importance of cattle and buffalo farming was truly realized with the advent of white revolution in India. The result of this enterprise is that, today India has the largest cattle (199.08 million) and buffalo (105.34 million) population in the world (Livestock Census, Directorate of Economics and Statistics and Animal Husbandry Statistic Division, Department Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture) and is largest producer of milk (127.3 million metric tons) in the world (Parekh,2013). However, beneath the veneer of this face of cattle farming in India lies the fact that the milk yield obtained from individual animal is still low compared to global standards. Thus one would be justified in saying that although great strides have taken in progress of animal husbandry especially cattle and buffalo farming, it still offers a great scope and potential for further development. This can be achieved by overcoming two important limiting factors *viz*. genetic constitution of milk breeds and preponderance of variety of infections, the list of which keeps expanding and shuffling with the time and changing socioeconomic scenario. Indeed, it is a pleasure to note that efforts are being made in this direction throughout the country to achieve this goal.

Among the different infections that are gaining importance throughout the globe, cryptosporidiosis came into forefront in the past two to three decades. Cryptosporidium, an obligate, intracellular, apicomplexan enteric protozoa was initially thought as a commensal with no significant consequence pertaining to health and production. Hence subsequent to its first report by Tyzzer in 1907 in mice (*Cryptosporidium muris*), no serious cognizance was taken by the scientific

community until it was found to be associated with diarrhoea in turkey (*C. meleagridis*) and calves (*C. parvum*) in the mid of 20th century. As a result of this, later on, numerous reports describing its occurrence in different species of host were published and today the parasite is known to infect more than 150 species of animals belonging to mammalian, avian, reptiles, amphibian and fish (Fayer and Xiao, 2008). Among 23 valid species of Cryptosporidium enlisted in the literature till date, eight *viz., C. parvum, C.andersoni, C. ryanae, C.bovis, C. hominis, C. felis, C. canis,* and *C. suis* have been recorded to occur in bovines and the first four of the list have been found to be most common worldwide. These four species have also been encountered in buffaloes. In India, cryptosporidiosis was reported for the first time in faeces of cattle by Nooruddin and Sarma (1987) and in human night soil by Mathan *et al*. (1985).

Bovine cryptosporidiosis, primarily a patent infection in young calves till they attain immunological maturity, is invariably associated with neonatal diarrhoea with higher morbidity than mortality, thus resulting into weight loss and delayed or stunted growth reflecting substantial economic losses. However, the infection is self limiting corresponding to attainment of immunological competence with advancing age. Apart from causing economic losses to livestock industry, *Cryptosporidium* spp. has also gained attention of scientific community as emerging zoonoses owing to their public health significance. Due to Co-existence of different species of Cryptosporidium in animal and human subjects in the same proximity, the efforts were taken to establish zoonotic link and number of species including *C. parvum* now have been proved to be zoonotic. Humans acquire the infection through various sources such as direct contact with animals, farm equipments and appliances in addition to ingestion of contaminated food and water. Thus it poses a serious occupational hazard to veterinarians, livestock supervisors, farm workers, agricultural labourers, plumbers, human doctors and hospital staff. However, cryptosporidiosis came into lime light due to its association with immunocompromized individuals which could lead to life threatening impact particularly in HIV/ AIDS patients (Ajjampur, *et al.,* 2007, Kulkarni *et al*., 2009) in developing as well as developed world. Owing to its ability to survive in contaminated surroundings for prolong period and cause outbreaks in susceptible population, it

has been included as List B pathogen for biodefence by Office International des Epizooties (OIE, 2008)

Broad based surveys of bovine cryptosporidiosis in different geographical regions are thus essential for generating base line epidemiological data which can be taken as benchmark in designing a comprehensive control plan to curb not only economic losses but also to minimize zoonotic risk. After scanning through the available literature, it was learnt that the information of the disease in bovines in Maharashtra is practically nil. In this context the present study was undertaken with following broad objectives in mind.

1. To note prevalence of *Cryptosporidium* spp. in cattle and buffalo calves in Mumbai region
2. To identify species of *Cryptosporidium* involved in the infection employing molecular tools
3. To assess zoonotic risk in relation to prevalence rate and occurrence of different species of *Cryptosporidium*

2. REVIEW OF LITERATURE

The study conducted in the project included three important aspects of Cryptosporidiosis *viz*., survey, molecular identification of species and zoonotic risk assessment and hence available literature is reviewed under the three sub headings in the following pages.

2.1 Survey of cryptosporidiosis:

Prasad *et al*. (1989) monitored 12 buffalo calves born during October 1984 to January 1986 in a dairy farm of Banaras Hindu University for prevalence of oocysts of *Cryptosporidium* spp in the faeces up to 28th day of birth. Faecal samples of each calf collected twice weekly were examined by modified acid fast and May-Grunwald Giemsa Technique. Eight out 12 buffalo calves developed diarrhoea during neonatal life and one buffalo calf excreted oocyst of *Cryptosporidium* spp in diarrhoeic faeces. Diarrhoea lasted for 6 days and oocysts were detected in the loose faeces during entire period of illness . The intensity of oocysts during first two days of illness was more than five per high power field which declined gradually over the period of next four days and eventually disappeared on 7th day. The authors claimed that the case under the report was the first evidence of Cryptosporidial diarrhoea in buffalo calves.

Banerjee *et al.* (2012) in their article reviewed status of Cryptosporidiosis in India and stated that 23 species of *Cryptosporidium* have been accepted as valid species which also included eight species from cattle *viz*., *C.parvum*, *C.bovis*, *C.andersoni*, *C.ryanae*, *C.hominis*, *C.felis*, *C.canis* and C*.suis,* identified on the basis of molecular analysis. First four species of *Cryptosporidium* have been reported most frequently not only from cattle but also from buffaloes. The author further stated that, although conventional methods such as concentration and differential staining of faecal smears with safranin methylene blue stain, DMSO carbolfuchsin stain, qinacrine, modified Ziehl Neelsen stain (mZN) , auramine phenol (AP) and Wright Giemsa stain and immunological techniques Like FAT, LAT, ELISA , RPHA etc. can be employed for diagnostic purpose, these do not differentiate *Cryptosporidium* infection at species and sub species level due to morphometric and antigenic similarity between most of the species.

According to the author, genetic analysis of the 18s SSU rRNA, heat shock proteins (HSP70), 60kda glycoprotein, *Cryptosporidium* oocyst wall proteins (COWP) and small double stranded virus like RNA gene has showed excellent results. Microsatellite and minisatellite analysis and multilocus sequence analysis have also been used for studying the parasite genotype.

The rate of occurrence of of Cryptosporidiosis in man and animals in Calcutta city was recorded by Chattopadhyay *et al*. (2000) during which a total 360 faecal samples from 159 cattle, 51 buffaloes, 50 dogs and 100 human beings belonging to two equal groups *viz* animal handlers (50), and non animal handler (50), were concentrated by sheathers sugar solution and stained by mZN technique for detection of oocysts. Prevalence of *Cryptosporidium* spp. was found to be 8.46% in animals with higher rate in cattle (11..32%) followed by buffaloes (7.85%) and it was nil in dogs. The occurrence was more in diarrhoeic cattle (14.55%) as compared to non diarrhoeic cattle (4.04%); however in buffaloes the oocysts were encountered only in diarrhoeic faeces (12.97%). Among positive animals, the prevalence rate was highest in cattle (14.02%) and buffaloe (11.54%) calves during neonatal life. The prevalence rate of Cryptosporidiosis in male cattle (12.68%) and buffaloes (11.54%) was distinctly higher than that of females (10.22% and 4.00%, respectively.) In the human stool samples the overall prevalence rate was found to be 3% and all the three cases were from the animal handler group indicating zoonotic significance. The oocysts were encountered in all the months except December and January.

Jaybal and Ray (2005) collected 30 faecal samples of cattle (15) and buffaloe (15) calves below three month of age housed at dairy farm of Indian Veterinary Research Institute and processed in the laboratory. The samples concentrated by sheather's sugar solution and the smears stained by modified Zeihl-Neelsen technique were screened under oil immersion for detection of oocysts of *Cryptosporidium* spp. which appeared as densely stained red bodies against the contrast green background. The size of oocysts varied from 2.8-3.1µm to 5.6-6.3um and showed varying number of dark blue or brownish internal bodies. The prevalence rate was found to be 33.33% in cattle and 53.33% in buffalo calves. There was no conspicuous difference in the prevalence rate of

infection in diarrhoeic and non diarrhoeic calves and in calves below 1 month and between 1-3 months age groups.

Roy *et al*. (2006) assessed the epidemiology of bovine Cryptosporidiosis over the period of two years in and around Kolkata during which they screened 470 faecal samples per year from diarrhoeic and non diarrhoeic bovines below one year of age by conventional method . *Cryptosporidium* spp. oocysts were encountered in 17.46 and 18.04% samples in the first and second year, respectively. The oocysts were present 50.21% and 49.79% diarrhoeic and non diarrhoeic faecal samples, respectively. Irrespective of the clinical status, the prevalence was highest in calves below one month ($P<0.01$). Highest prevalence was recorded in monsoon (27.55%) followed by that in summer (16.99%) and winter (8.71%) season ($P<0.01$). A total of 166 positive cases were subjected to molecular characterization to ascertain the species of Cryptosporidium involved in the infection by employing PCR-RFLP analysis of SSU rRNA gene. The investigation revealed *C.parvam* as the predominant species responsible for diarrhoea in bovines.

Santin *et al*. (2008) conducted longitudinal study of Cryptosporidium in dairy cattle from birth to two years of age in Marylad, USA during which faecal samples of thirty individuals were periodically collected and processed by immunoflurosence machinery and PCR to determine the age wise prevalence of cryptosporidium. All thirty individuals were found to shed the oocysts at some time during first two years of life. The highest prevalence rate was noted at age of 2 weeks when 29 out of 30 calves were found excreting oocysts. Thus, the prevalence was highest in pre weaned calves upto 8 weeks of age (45.8%) followed by in post weaned calves of 3 to 12 months of age (18.5%) and it was lowest in heifers of 12 to 24 months (2.2%). Sequence analysis of 190 PCR products identified *C.parvum, C.bovis, Cryptosporidium deer like genotype, C.andersoni* with their cumulative prevalence reading 100%, 80%, 60% and 3.3%, respectively.

Mallinath *et al*. (2009) Made some preliminary investigations on occurrence of cryptosporidiosis in bovines in five organized dairy farms located in and around Bangalore and data were analyzed with respect to age, sex, breed

of the animal to draw relevant conclusions. A total of 455 faecal samples collected from five different organized dairy farms and veterinary hospitals were subjected to Sheather's sugar floatation method for detection of the oocysts. The prevalence was found to be 5.71% . The species identified on the basis of morphology and morphometery of the oocysts by three staining procedures *viz* mZN, Kinyoun's and safranine methylene blue, to detect *C.parvum* and *C.andersoni*. The occurrence and intensity of Cryptosporidiosis were highest in calves less than a month (9.25%) as compared to older animals (2.66 to 4.71%). The frequency of appearance of oocysts was higher in diarrhoeic (6.99%) as compared to non diarrhoeic (3.83%) faeces. The oocysts encountered only in the faeces of crossbred animals (6.58%) and none of the 60 faecal samples from Deoni, Surti,and Non Descript animals were found positive. In crossbred group the prevalence rate was higher in HF (6.77%) than Jersey (5.88%) crosses. The oocysts were commonly encountered in faecal samples of female (6.0%) as compared to male (3.64%) animals. The differences in the prevalence rate with respect to age, sex, breed and clinical status of the host were found to be statistically significant ($p \leq 0.05$). The morphometric analysis revealed 10.8% prevalence of *C.parvum*, 5.9% prevalence of *C.andersoni* and 5.98% prevalence of mixed infection. The oocysts of *C.andersoni* measured 7.2±0.835×5.7±0.835 um while that of *C.parvum* measured 5.2±0.42× 4.05±0.052µm. Faecal samples treated with sheathers sugar solution demonstrated round to oval refractile bodies with granular cytoplasm and prominent black dot. Stained faecal smears revealed pink to red colored oocysts with majority of them showing clear hallo. Very few of the stained oocysts showed sprozoites arranged at periphery.

Maurya *et al*. (2011) investigated calfhood diarrhoea in an organized dairy farm from September to November 2010 during which loose faecal samples collected per rectally from 19 cattle and 21 buffalo calves less than one month of age were processed by modified Ziehl Neelsen acid fast staining technique. Out of 40 samples,14 (35%) including 5 (26.32%) from cattle and 9 (42.86%) from buffaloes showed oocysts of *Cryptosporidium* spp. Further, amongst the various enteric pathogens including *Emeria bovis, E. zuernii, E.bareillyi* (only in buffalo) the prevalence of Cryptosporidiosis was higher.

Rana *et al*. (2011) noted prevalence of gastrointestinal parasites in neonatal buffalo calves at an organized herd in Haryana for which a total of 142 faecal samples collected directly from rectum were processed by floatation (saturated salt solution) and modified Ziehl Neelsen techniques. Cryptosporidium oocysts measuring 4.8 x 5.2µm were found in five (3.52%) calves.

Veena *et al*. (2011) subjected faecal and sera samples of 201 cattle from different farms, animal shelters and abattoirs in and around Bangalore to sheathers sucrose floatation and indirect fluorescent antibody test to detect oocysts and antibodies of *Cryptosporidium* spp., respectively. Faecal samples of 11 (5.5%) individuals revealed oocysts of Cryptosporidium which were identified on the basis of morphology and micrometry. *C.parvum* oocysts were seen in two samples, and *C.andersoni* oocysts were encountered in six samples remaining 3 samples had mix infection by both the species.

Yadav *et al*. (2011) recruited conventional, immunological and molecular tools for identification of Cryptosporidium oocysts in bovine calves faecal samples collected in Jammu district. Prevalence recorded by PCR, antigen ELISA and mZN was found to be 36.11%, 23.9% and 14.89%, respectively. The prevalence was significantly higher in cattle (26.18%) calves as compared to buffalo calves (16.45%). The highest prevalence was recorded in calves below one month of age and the incidence found to be decreasing with advancing age. Similarly occurrence rate was higher in diarrhoeic calves (30.53%) than non diarrhoeic calves (15.25%), in male (28.23%) as compared to female host (18.85%), in monsoon (33.19%) as compared to the other two seasons and in crossbreed (32.39%) than indigenous (16.15%) calves. The intensity of oocysts was higher in diarrhoeic calves and particularly in monsoon. The author's recommended that modified Ziehl Neelsen method should be included in routine diagnostic protocol particularly for the young individuals.

Prevalence of Cryptosporidiosis in five different organised dairy farms located in and around Bangalore was studied by Rekha *et al*. (2011) by performing sheathers sugar floatation method in 455 faecal samples. The prevalence rate was found to be 5.71% with higher rate of positivity in diarrhoric samples. Negative staining methods, *viz.,* Negrosin and Malachite green, were

used for detection of *Cryptosporidium* spp. oocysts which appared as refractile unstained minute bodies against black or green background, respectively. In both the methods although the internal structures were not visible, a single prominent dot was seen in few oocysts. The authors opined that malachite green gave better result as differentiation between parasite and yeast was possible.

Bhatt *et al*. (2012) investigated per rectally collected 162 faecal samples of buffaloe calves of either sex below five months of age residing at different dairy farms situated at periurban surroundings of Ludhiana, Punjab for presence of oocysts of Cryptosporidium by employing modified Ziehl-Neelsen method from June 2009 to May 2010. Positive samples were subjected to nested PCR for confirmation. Apple red coloured oocysts of *Cryptosporidium* spp. against pale green coloured background were seen in 62 (38.30%) faecal samples. The prevalence rate was higher (49.30%) in diarrhoeic samples as compared to that in non diarrhoeic (28.13%) samples. As regards seasonal influence, the occurrence was marginally higher in monsoon (40.35%) as compared to other seasons. The frequency of occurrence of the oocysts was highest in calves during first month of life (65.71%) and as the age of the host advanced thereafter there was gradual decline in the frequency of occurrence , lowest being 5.88% in calves between 4 and 5 months of age. Prevalence of cryptosporidiosis was higher in female (40.35%) than male (30.30%) calves. Good correlation between conventional and molecular methods was observed.

Influence of host related factors such as age, sex, breed, dung consistency and management related factors such as " rearing system " on prevalence of cryptosporidiosis in south Indian cattle was studied by Venu *et al*. (2012). Two steped nested PCR was employed for detection of Cryptosporidiosis in dairy calves of Andhra Pradesh, Karnataka, Kerala, Tamil Nadu and Pondicherry. The overall prevalence was found to be 39.65%. Occurrence of Cryptosporidiosis was higher in young calves below one month of age and in female calves as compared to male calves. Based on consistency, the oocysts were commonly encountered in dung having semisolid consistency followed by that in well formed and diarrhoeic faeces. Prevalence rate was lower in calves maintained in well organized farms as compared to the calves reared under small household backyard system.

Yadav *et al*. (2012) made preliminary investigations on Cryptosporidiosis in calves in private dairy farms in Jammu. Faecal samples processed by modified Ziehl- Neelsen method revealed positivity of 32.68%. Highest prevalence rate was found in calves below one month age group and it was found decreasing thereafter with increasing age. Further, occurrence of Cryptosporidium oocysts was higher in calves showing diarrhea (48.30%) as compared to apparently healty calves (12.64%). Intensity of infection noted as per OIE standards showed higher load of oocyst in diarrhoeic stools. Sex-wise and season-wise analysis of data revealed higher prevalence rate in males as compared to females and in winter season (56.48%) as compared to monsoon and summer (7.28%). The difference in the prevalence rate between crossbred (38.46%) and indigenous calves (12.7%) was statically significant ($P<0.05$).

2.2. Molecular Identification of bovine Cryptosporidiosis:

Although conventional diagnostic procedures such as modified Ziehl-Neesen staining of faecal smear was considered as “Gold Standard ” for preliminary diagnosis of Cryptosporidiosis, species identification is mainly based on molecular methods. The literature accumulated in this regard is reviewed in following paragraphs.

Venu *et al*. (2011) attempted molecular typing of *Cryptosporidium* spp. in dairy calves of south India by extracting genomic DNA from dung samples and subjecting them subsequently to nested PCR using 18 subunit rRNA gene fragment primer. Electrophoretic analysis of primery PCR product revealed distinct band at 1325 bp in 24 (5.23%); whereas secondary amplicon of 830 bp was observed in 182 (39.54%) dung samples. Detection limit of nested PCR was as low as 100 oocysts per samples. Out of 182 nested PCR products, 64 were genotyped using *SspI, VspI* and *MboII* restriction enzymes and the exercise revealed *C.andersoni, C. ryanae, C.parvum and C.bovis* in 39(60.91%) ,18(28.13%), 4(6.25%) and 3 (4.69%) samples, respectively. DNA sequence analysis 19 samples revealed *C.andersoni, C.ryanae, C.parvum* ,*C.bovis* and *C.ryanae* in 9 (47.37%) ,8 (42.11%),1 (5.26) and 1 (5.26%), respectively. Both, PCR-RFLP and DNA sequencing showed excellent correlation.

Xiao *et al*. (1999) stated that extensive diversities were present among *C.parvum* isolates from human, calves, pigs, dogs, mice, ferrets, marsupials and monkeys. Their observation was based on molecular study of isolates of *Cryptosporidium parvum* from the eight hosts exploiting SSU rRNA gene. The authors recommended that, assessment study of zoonotic potential of *Cryptosporidium parvum* isolates of animal origin is necessary to ascertain the public health importance.

Agnihotri *et al*. (2012) opined that, although several diagnostic procedures are available for detection of clinical and sub clinical cases of cryptosporidiosis in variety of host , molecular tools *viz,* PCR, nested PCR and PCR-RFLP are very useful for improving not only sensitivity but also specificity up to species level and thus are of genuine utility in epidemiological studies.

Feng *et al*. (2007) in multinational study, based on molecular investigation of faecal samples of 6 calves from shanghai (china), 12 calves from Kolkata (india) and 32 calves belonging to three farms from Georgia (USA) attempted identification of the species of Cryptosporidium involved in the infection. RFLP-PCR using three different restriction enzymes *viz*., *VspI*, *SspI* and *MboII* revealed that, out of the 12 samples from India, 11 had *C.bovis* and remaining one had *C.parvum* infection.

Rakesh *et al*. (2012) extracted DNA of *Cryptosporidium* spp. from faecal samples of 10 neonatal bovine calves including 6 cattle and 4 buffaloes which revealed the oocysts microscopically when processed with modified Ziehl Neelsen staining technique. The DNA of 18S rRNA gene was subjected to nested PCR which produced an amplicon of 834 bp on agarose gel. RFLP analysis of Nested product using *SspI, VspI*, and *MboII* confirm *C.parvum* as the only prevailed species. Cloning, sequencing and sequence analysis of 834 bp of 18S subunit gene confirmed the presence of *C.parvum.*

Venu *et al*. (2012) assessed *MboII, SspI* and *VspI* enzymes in RFLP in detection of four major *Cryptosporidium* species *viz, C.parvum, C.ryanae* ,*C.andersoni* and *C.bovis* by exploiting 18 subunit RNA in nested PCR. *SspI* alone was found adequate to differentiate *C.andersoni* from other species by

unique RFLP, while *MboII* could differentiate *C.bovis, C.ryanae* and *C.parvum.* The authors concluded that, *MboII* in combination with *SspI* provides economical alternate to sequencing of Cryptosporidium of dairy cattle.

Khan *et al*. (2010) in an attempt to establish zoonotic link between cattle and humans, examined 180 bovine and 51 farm worker faecal samples from two dairy farms at Kolkata between October 2008 and August 2009 by employing modified kiyouns acid fast staining method and 18 subunit rRNA based nested PCR method. The overall prevalence of cryptosporidium in cattle was 11.7%. Age wise split-up of data revealed highest rate in pre weaned calves (20%) followed by that in post weaned calves (13.9%) and heifers or adults (4.4%). Correspondingly, 11.8% of farm workers were found positive for oocysts of *Cryptosporium* spp. Primary and secondary PCR analysis revealed 1325 bp and 830 bp fragment lengths, respectively. RFLP analysis of PCR products with *SspI, VspI*, and *MboII* revealed *C.parvum, C.bovis , C.rayanae* and *C.andersoni.* Different species of Cryptosporidium have different age prevalence as *C. parvum* found only in preweaned calves, *C.bovis* and *C.ryanae* were detected mostly in postweaned group and *C.andersoni* was found mostly in heifers or adult age group. In farm workers, *C.hominis* was most commonly encounted species followed by *C.parvum* and in one worker noval *C.bovis* genotype was detected showing a mismatch of one base pair (G-A) at 491 position.

2.3. Pubic health Importance of bovine Cryptosporidiosis:

In over populated cities like Mumbai, where hygienic aspects are compromised owing to ever-growing population, there is every possibility of zoonotic pathogens taking upper hand in crossing over from animal to human and vis-a versa. The infection, usually non pathogenic, takes clinical course in immunocompramised individuals particularly in HIV subjects. The literature on public health aspect is reviewed briefly as under

Lanjewar *et al*. (1996) compared spectrum of gastrointestinal infections associated with AIDS patients in India with that in western countries. The study based on autopsy of 49 including 35 diarrhoeaic and 14 non diarrhoeic subjects from Mumbai conducted between 1998 and 1993 demonstrated Cryptosporidium

infection in 10% of cases and 80% of them had diarrhoea before death. The parasites located either in duodenum or colon were seen as very small (2-3µm), haematoxylinophilic bodies attached to the mucosal surface of the epithelial cells.

Sulaiman *et al*. (1999) compared genotyping techniques for *Cryptosporidium* spp. during which they evaluated sensitivity and specificity of 11 previously described species differentiation and PCR protocols. Genomic DNA from three species of Cryptosporidium (genotype1 and genotype 2 of *C.parvum, C.muris, C.serpentis*), two species of Eimeria (*E.neischutzi* and *E.papillata*) and *Giardia duodinalis* were used to evaluate specificity of primers. Out of the 11 protocols studied, 10 amplified *C.parvum* genotype 1 and 2 and the expected fragment sizes were obtained. The study revealed that two species differentiation protocols were not Cryptosporidium specific as the primers employed in these protocols also amplified DNA of *Eimeria* spp. Thus two nested PCR restriction fragment length polymorphism (RFLP) protocols based on the small subunit rRNA and Dihydrofolate Reductase genes were found more sensitive than single rounds of PCR or PCR-RFLP protocols. The authors found that PCR amplification techniques that targeted genes encoding oocyst wall protein, the small subunit of rRNA, β tubulin, TRAP-C1, TRAP-C2, ITS1, Polythreonine repeat (poly-T), Dehydrofolate reductase (DHFR) and rRNA of heat shock proteins have been successful in detection and differentiation of *Cryptosporidium* spp.

Chawdhary and joshi (2002) determined spectrum of parasitic infections in 94 AIDS patients showing chronic diarrhoea between October 1994 and December 1996. The faecal samples processed by direct wet mount microscopy of saline and iodine preparation by modified (kinyoun's) Ziehl-Neelsen stain revealed Cryptosporidium infection in 11% of patients in Mumbai. The authors remarked that *Cryptosporidium* spp. is one of the most common opportunistic infections in AIDS or HIV subjects.

Dalvi *et al*. (2006) conducted a prospective study on 64 HIV seropositive individuals with diarrhoea in Mumbai from October 2004 to September 2005, to identify the parasitic agents. The analysis based on modified Zeihl-Neelsen

method showed 3.12% prevalence of Cryptosporidisis in the studied group of individuals. Both the stool samples that tested positive had watery consistency.

Roy *et al*. (2006) owing to inadequate information about zoonotic status of *Cryptosporidium* spp in India recommended molecular epidemiological studies to know current scenario of the disease in different parts of the country. The authors conducted a small study in West Bengal in 2003-04 during which the prevalence was found to be 18.51±3.45 and 6.68±1.72 in diarrhoeic and non diarrhoeic bovines, respectively and the difference was statistically significant ($p<0.01$). Diarrhoeic and non diarrhoeic human species revealed prevalence of 4.62 and 1.24% with overall rate of 2.99% in the same region indicating zoonotic link.

Nagamani *et al*. (2007), with an objective to understand epidemiology of Cryptosporidiosis in twin cities of Hyderabad and Secunderabad, conducted a survey based on examination of faecal samples from 681 children and 804 adult population having preliminary complaint of diarrhoea. In addition faecal samples of six calves with diarrhoea were also screened for presence of oocysts of *Cryptosporidium* spp. The infection was detected in 7.6% of children, 0.9% of adult population and 16.6% of the calves. Amongst the children, prevalence rate was higher (14.3%) in the age group of six months to one year and the rate gradually declined with the advancing age. Out of 42 samples genotyped , 29 (69%) were identified as *C.hominis* and 8 (19%) had *C.parvum* infection and remaining five (11.9%) samples had mixed infection involving both the species. The authors concluded that, occurrence of *C.parvum* in almost one third of samples adequately suggests zoonotic transmission.

Ajjampur and Kang (2008) published an overview on *Cryptosporidium* spp. in HIV infected individuals in India. The compilation of reports on Cryptosporidium in HIV infected adults from different parts of India revealed prevalence rate of 7.83% in symptomatic and 1.4% to 5.7% in asymptomatic individuals. Among children with diarrhoea, the prevalence of *Cryptosporidium* spp.ranged from 1.1 to 18.9%. Apart from HIV group, other susceptible populations include patients with malignancies and transplant recipients. The review article also revealed occurrence rate of 8.5 to 31%. among symptomatic HIV patients residing in Mumbai.

Chhabra and Singla (2009) in there review article on food borne parasitic zoonosis in India, emphatically remarked that Cryptosporidiosis contributes significantly to cause human diarrhoeal disease worldwide. The parasite has been recognized as the third commonest microbial infection in AIDS.

De *et al.* (2009) investigated stool sample of 140 HIV positive Individuals including 100 patients with and remaining 40 patients without diarrhoea for detection of opportunistic parasitic infection. Out of 13 samples tasted positive by modified Ziehl-Neelsen method, liquid stool was seen in 69.2% cases and majority of them had 6-10 motions per day.

Singh *et al*. (2010) found that cryptosporidiosis is an emerging zoonotic protozoan parasite of young bovines and was associated with diarrhoea in children. The infection was reported to be significantly higher in urban slums, diarrhoeic patients and patients with HIV.

Ujagare *et al.* (2012) processed stool samples of 490 individuals in Maharashtra, including 110 HIV reactive and 380 HIV non reactive, with four different stool concentration technique. Cryptosporidium oocysts were detected only in 4 stool samples from HIV reactive group and all the four individuals had watery diarrhoea highlightining opportunistic feature of the organisms.

Kumar *et al*. (2004) highlighted public health significance of animal Cryptosporidiosis throughout the world. Among different species encountered in animals,.*C.parvum* was rated to be the most serious human and livestock pathogen responsible for significant morbidity in immunocampetent and mortality in imunocompramized individuals. Low infective dose, small size of oocyst, shedding of large number of oocysts, poor efficacy of commonly used coccidiostat, and antibiotics were considered to be the factors responsible for widespread occurrence of the infection.

3. MATERIALS AND METHODS

3.1. Collection of faecal samples:

Present study on prevalence of Cryptosporidiosis in cattle and buffaloes particularly at very young age was carried out in and around Mumbai from November 2012 to June 2013. For demonstration of oocysts of Cryptosporidium faecal samples of cattle and buffalo calves of either sex aging below three months were collected per rectally using separate gloves for each animal in 50 ml sterile plastic vials. Few samples from adult cattle and buffaloes were also collected randomly in similar fashion. All the vials were immediately transferred to laboratory of Department of Veterinary Parasitology, Bombay Veterinary College for further processing to demonstrate the oocysts. Few representative faecal samples containing the oocysts were subjected to molecular analysis to identify the species of Cryptosporidium involved in the infection.

3.2. Processing of faecal samples in laboratory:

In the laboratory faecal samples were subjected to two types of techniques *viz*.,I) conventional methods for detection of oocysts and ii) molecular method for identification of DNA of Cryptosporidium.

3.2.1 Conventional methods for detection of oocyst of *Cryptosporidium* spp:

The samples were handled at designated place and all the objects came in contact with the samples were decontaminated immediately by immersing in 20% formalin and in 30% ammonia solution for 24 hrs. In all 391, faecal samples including 125 from cattle calves, 218 from buffalo calves, 20 from adult cattle and 28 from adult buffaloes, were processed by four different methods to demonstrate the oocysts of *Cryptosporidium* spp.

3.2.1.1. Direct faecal smear examination (DFSE):

Faecal smears were made on grease free microscopic glass slides with the help of non glue end of match stick. After drying, smears were stained by modified Ziehl-Neelsen (mZN) technique (Henricksen and Pohlenz,1981). The air dried smears were fixed with methanol for five minutes during which the fixative

was allowed to evaporate. The smears were then transiently fixed over the flame. Subsequently the smears were kept on the rack and flooded with carbol fuchsine which was allowed to act for 40 minutes. The slides washed under running water for 5 minute were then decolourised with 10% H_2SO_4 for 15-30 seconds after counter staining them with 5% malachite green for 5 minutes. Slides washed under running water for five minutes were dried and examined systematically under high (45x) and oil immersion (100x) objectives of compound microscope.

3.2.1.2. Faecal smear examination after NS sedimentation (NSS):

Approximately five grams of faecal matter was homogenised in 10 ml normal saline solution (NSS) in a glass beaker with the help of a glass rod . The faecal suspension was sieved through a wire mesh of 400µ pour size to eliminate course particles. The suspension was then centrifuged at 3000 rpm for five minutes. The supernatant was discarded gradually by tilting the tube and sediment was used to prepare a faecal smear which was subsequently stained with mZN as per the procedure described earlier (3.2.1.1). The smears were examined systematically under high power and oil immersion objectives of compound microscope.

3.2.1.3. Unstained faecal smear examination after sheathers floatation (SF):

Faecal samples were homogenised in phosphate buffer saline (PBS), sieved through wire mesh (400µ) and centrifuged at 3000 rpm for five minutes. After discarding the supernatent the sediment was mixed thoroughly with 10 ml of sheather's sugar solution in the centrifuge tubes which were centrifuged at 2000 rpm for 10minutes (Anderson,1981). The superficial film of suspension was collected gently on a clean grease free microscope slide and cover slip was placed on it. The slide was examined under high power (45x) objective compound microscope.

3.2.1.4 Stained faecal smear after sheathers flotation (SFSS):

The method employed here was same as SF (3.2.1.3) up to centrifugation in sheather's sugar solution. Subsequently, 2-3ml of top layer was aspirated in clean sterilised pasture pipette and it was diluted three times with PBS

(Phosphate Buffer Saline) and centrifuged at 4000 rpm for five minutes. After discarding the supernatant, the sediment was used to prepare the faecal smear on clean grease free microscope slide and on drying it was processed in similar fashion as described earlier (3.2.1.1). The smears were searched for presence of oocysts of Cryptosporidium under high power and oil immersion objectives of compound microscope. Oocysts encountered during microscopic examination of the stained smears were measured by using micrometry technique (Rathore and Senger, 2005). In each smear, at least 10 oocysts were measured under high power.

3.2.2. Molecular analysis of stool samples:

Nine representative samples showing positive results during conventional technique were subjected to molecular analysis in an effort to identify the species of Cryptosporidium involved in the infection.

3.2.2.1 Reagents:

70% analytical grade ethanol
95% analytical grade ethanol

3.2.2.2 Kits:

1. QIAmp DNA stool mini kit (Qiagen, Germany) used to extract parasitic DNA from the oocysts in stool samples.
2. DNA amplification core kit (Bangalore Genei, India) used for amplification of the genomic DNA
3. Nucleic acid agarose gel electrophoresis kit (Bangalore Genei, India) used for electrophoresis of PCR products.

3.2.2.3 Glass and plastic-wares:

All glass and plastic-wares purchased from Borosil and Tarsons were sterilized in hot air oven and autoclave, respectively, before use.

3.2.2.4 Scientific equipments:

1. Microcentrifuge (Spinwin)
2. Vortex (Remi)
3. Laminar flow
4. Thermal cycler (Techgene, Cambridge, U.S)
5. Microwave
6. Incubator
7. Submarine gel electrophoresis apparatus
8. Micro pipettes
9. U.V. Transilluminator

3.2.2.5 Nested PCR:

3.2.2.5.1 Purification of oocysts

Oocyst of *Cryptosporidium* spp. purified from faecal mass were used for extraction of DNA.Primary purification of the oocysts from faeces was done by modified sheather's sucrose floatation (Current *et al*., 1983) technique. About 5 gms of faeces was mixed thoroughly with an equal volume of 2.5% potassium dichromate solution (w/v) and sieved initially through a wire mesh of 400µ pore size and then through 200µ pore size. The faecal sample was then mixed with an equal volume of diethyl ether in a 15 ml centrifuge tube which was shaken vigorously. The sample was centrifuged at 2000 rpm for 10 minutes. The fat plug and other fat soluble materials in the upper organic/aromatic layer were discarded. The lower aqueous phase was taken in another 15 ml centrifuge tube, mixed with equal volume of 2.5% potassium dichromate solution ($K_2Cr_2O_7$) and centrifuged at 5000 rpm for 15 minutes. The sediment was taken and resuspended in 1 ml of 2.5% potassium dichromate solution mixed with 0.2% Tween-20. The one milliliter of faecal suspension was then mixed with 9 ml of modified sheather's sugar solution in a 15 ml centrifuge tube and it was centrifuged at 5000 rpm for 10 minutes. One ml of 0.2% aqueous solution of Tween- 20 was layered over the floatation medium. The top layer of the mixture was stirred carefully with a pipette and about 1 ml was aspirated with it. The remaining material was again centrifuged. This step was repeated several times and oocysts harvested each time were monitored under compound microscope. Equal volume of aqueous solution of 0.2% Tween-20 was added to the harvested

oocyst suspension and centrifuged at 5000 rpm for 15 minutes. The pelleted oocysts were resuspended in antibiotic solution and stored at 4^0C for DNA extraction. Purified oocyst were subjected to QIAmp® DNA stool extraction kit according to manufacturer's protocol with few modifications.

3.2.2.5.2 Isolation of genomic DNA:

The concentrated oocysts sediment were used for the extraction of DNA with the help of QIAamp® DNA Stool Extraction kit according to manufacturer's protocol with few modifications.

1. Two hundred milligram of oocyst pellet was taken in a 2 ml microcentrifuge tube and placed it in ice.
2. Buffer ASL (1.4 ml) was added to each sample which was vortexed continuously for 2 minutes or until it was thoroughly homogenised.
3. Suspension was heated for 12 hrs at 70^o C in waterbath.
4. The tube was vortexed for 15 seconds and centrifuged at full speed for two minutes to form pellet.
5. The supernatant was pipetted into a new 2 ml microcentrifuge tube and the pellet was discarded.
6. A Inhibit Ex tablet was added to each sample and vortexed immediately and continuously for 1min or until tablet was completely suspended. The suspension was incubated for five minutes at room temperature to allow inhibiters to absorb to the InhibitEx matrix.
7. The samples were centrifuged at full speed for five minutes to bound pellet inhibitors to InhibitEx matrix.
8. The supernatant was pipetted into new 1.5 ml microcentrifuge tube and the pellet was discarded. The sample were centrifuged at full speed for 3min.
9. Protinase k (15µl) was pipetted into a new 1.5 ml microcentrifuge tube.

10. The supernatant (200ul) from step 8 was pipetted into the 1.5ml microcentrifuge tube containing protinase k.

11. Buffer ASL (200µl) was added and vortexed for 15 seconds.

12. The tube was incubated at 70^{o} C for 30 min.

13. Two hundred microlitre of ethanol (96%) was added to lysate and mixed by vortexing.

14. The lid of new QIAmp spin column was labelled and placed in a 2ml collection tube. Complete lysate from step 13 was pipetted to the QIAmp spin column without moistening the rim. The cap was closed and centrifuged at full speed for 2 min. The QIAmp spin column was then placed in a new 2ml collection tube and the tube containing filtrate was discarded.

15. The spin column was opened carefully and 500ul buffer AW1 was added. The cap was closed and the column was centrifuged at full speed for 2 min. The spin column was then placed in a new collection tube and the collection tube containing filtrate was discarded.

16. The spin was opened carefully and 500ul buffer AW2 was added. The cap was closed and centrifuged at full speed for 3 min. The collection tube containing filtrate was discarded.

17. The spin column was placed in a new collection tube and the old collection tube with the filtrate was discarded . The tube was centrifuged at full speed for 1min.

18. The spin column was transferred into a new, labelled 1.5ml microcentrifuge tube .The spin column was opened carefully and 50ul buffer was pipetted directly onto the QIAmp membrane. The cap was closed and incubated for 1 min at room temperature before centrifuging at full speed for 1 min to elute DNA.

19. For long term storage eluted DNA was kept at -20^{o} C.

3.2.2.5.3. Quantification of DNA:

Quantification of DNA extracted from faecal samples was done spectrophotometrically at 260 nm and 280 nm. The $OD_{260:280}$ ratio was calculated.

3.2.2.5.4 Agarose gel electrophoresis:

The purity of DNA was verified by agarose gel electrophoresis in a submarine horizontal electrophoresis apparatus. Agarose gel (1%), prepared by boiling agarose powder in a measuring volume of 1X TAE buffer, was poured on a gel casting tray fitted with comb, which was removed after solidification of the gel. Extracted DNA suspension of 7ul was mixed with 2ul of 6x loading dye and loaded into the wells . Electrophoresis was run at 2-3volts/cm or at (40V) and the mobility was monitored by migration of the dye. The gel was then visualised in a gel documentation system and data were recorded.

3.2.2.5.5 Amplification of 18s SSU rRNA gene of C*ryptosporidium* spp:

The oligonucleotide primers described by Xiao *et al.* (1999) were used in this study.The primers obtained in lyophilized form were resuspended in autoclaved water and a working solution of 20 pmole/ µl was made. One µl of the working solution was used in 25µl PCR mixture. The nested PCR protocol described by Xiao, *et al.* (1999) was followed with minor modifications.

Name of Primers	Oligonucleotide sequence
Primers for primary PCR	
CRP-DIAG1 Forward primer:	5' TTCTAGAGCTAATACATGCG 3'
CRP-DIAG1 Reverse primer:	5' CATTTCCTTCGAAACAGGA 3'
Primers for secondary/nested PCR	
CRP-DIAG2 Forward primer:	5' GGAAGGGTTGTATTTATTAGATAAAG 3'
CRP-DIAG2 Reverse primer:	5' AAGGAGTAAGGAACAACCTCCA 3'

3.2.2.5.5.1 Standardization of primary PCR:

In order to amplify the 18 small sub-unit (SSU) rRNA gene of *Cryptosporidium* spp. by PCR, the following protocol was standardized. PCR master mixture (25µl) was composed as below:

Sr. No.	Contents	Quantity
1.	Genomic DNA	8.0 µl
2.	CRP-DIAG 1 F_1	1.0 µl
3.	CRP-DIAG 1 R_1	1.0 µl
4.	10x buffer without $MgCl_2$	2.5 µl
5.	$MgCl_2$ (25 mm)	2.0 µl
6.	dNTP (10mm)	0.5 µl
7.	Taq polymerase	0.5 µl
8.	Autoclaved distilled water	9.5 µl
	Total PCR mix	**25.0 µl**

The reagents listed above were added in serial order to prepare master mixture in eppendrof tube. Master mixture (25 µl) was taken in each PCR tube to which 2µl of template (DNA extract) was added (outside the laminar flow).

The cycling conditions used for amplifying the 18s SSU rRNA gene of *Cryptosporidium* spp were as follows:

Step 1: Denaturation 94^0C for 5 minutes

Step 2: Denaturation 94^0C for 1 minute
Annealing 56^0C for 1 minute
Extension 72^0C for 1 minute
(No. of cycles: 35)

Step 3: Final extension 72^0C for 10 minutes

Step 4: Held at 4°C for 10 mintutes

The amplification of specific PCR product was checked by gel electrophoresis of the PCR product in 1.5% agarose and viewed in UV Transilluminator system

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3.2.2.5.5.2 Standardization of secondary/nested PCR:

1ul amplicon of primery PCR product was used as template in secondary PCR. The 25 µl PCR master mixture was composed as below:

Sr. No.	Contents	Quantity
1.	Template	1.0 µl
2.	CRP-DIAG $2F_1$	1.0 µl
3.	CRP-DIAG 2 R_1	1.0 µl
4.	10x buffer without $MgCl_2$	2.5 µl
5.	$MgCl_2$ (25 mm)	1.0 µl
6.	dNTP (10mm)	0.5 µl
7.	Taq polymerase	0.5 µl
8.	Autoclaved distilled water	17.5 µl
	Total PCR mix	**25.0 µl**

The cycling conditions used for amplifying the 18s SSU rRNA gene of *Cryptosporidium* spp. were as follows:

Step 1: Denaturation 94^0C for 5 minutes

Step 2: Denaturation 94^0C for 1 minute
Annealing 57^0C for 1 minute
Extension 72^0C for 1 minute
(No. of cycles: 35)

Step 3: Final extension 72^0C for 10 minutes

Step 4: Held at 4°C for 10 mintutes

The amplification of specific PCR product was checked by electrophoresis of the PCR product in 1.5% agarose gel and viewed in UV Transilluminator system

3.2.2.5.5.3 Detection of PCR product by gel electrophoresis:

The agarose gel (1.5%) was prepared by adding 300 mg of agarose in 40 ml 1X TAE PCR electrophoresis buffer which was heated in microwave. one µl of ethidium bromide (containing 0.5 µg) was added in the molten agarose at the point when fumes disappeared. Electrophoresis apparatus was arranged and 20 ml of molten agarose was poured on the tray and the comb was placed at the anode end. After solidification of the agar, the comb was removed gently. five microlitre of amplified product was mixed with loading dye on parafilm and added in the respective wells. A 100 bp DNA ladder was run along with the samples during each electrophoresis.

3.2.2.6 Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) of the 18s SSU rRNA gene of *Cryptosporidium* spp.:

The PCR products revealing band at 834 bp were further subjected to RFLP. For genotyping, 9 nested PCR positive products were subjected to PCR-RFLP using the restriction enzymes *viz*., *SspI*, *Vsp*I and *MboII* (fermentas) following the manufacturer's instructions and protocol.

Five microlitre of the eluted PCR product (834 bp) was separately subjected to the restriction endonucleases enzymes *Ssp*I, *Vsp*I and MboII in 10ul reaction mixture, in 0.2 ml thin walled tubes for 8 hrs in a humid chamber.

The 10ul reaction mixture composed of

Eluted amplicon (DNA)	5.0 ul
10 x RE buffer	1.0 ul
Enzyme (*Ssp*I/ *Vsp*I/MboII)	0.5 ul
Water up to	10 ul

The confirmation of the digested product was made by electrophoresis of the product in 3% agarose gel and viewed in UV Transilluminator system.

3.2.2.7 Sequencing of 18s SSU rRNA gene of *Cryptosporidium* spp.:

The sequencing of the PCR products was carried out at Xcearis labs pvt Ltd, Ahmadabad, (India). The sequences obtained from the ABI files were analyzed and curated using chromas light software version 2.01.

3.2.2.7.1 Analysis of sequences using bioinformatics tools:

The sequences of amplicons of 18s rRNA gene were analysed using bioinformatic tools available online. Sequences retrieved from sdsc biology workbench (http//.workbench.sdsc.edu) ABI files were subjected for correct annotation using chromaslite software version 2.01. The curated sequences were submitted to search their similarity in NCBI database using BLAST tool.

3.3 Statistical analysis:

The data generated during the study with repect to different groups *viz*, cattle vs buffaloes, calves vs adults, diarrhoeic vs non diarrhoeic,male vs females samples were subjected to square test as per method described by Snedecor and Cochran (1994).

4. RESULTS AND DISCUSSION

Present study was conducted to note the prevalence of cryptosporidiosis in cattle and buffalo in Mumbai region. An attempt was also made to identify the species of Cryptosporidium involved in infection, on the basis of which public health significance of cryptosporidiosis in bovines was analysed. The findings of these aspects are dicussed in the light of available literature to draw relevant conclusions.

4.1 Prevalence of bovine cryptosporidiosis in Mumbai (Tables 1-5, Fig. 1-5 & Plate 1)

Different methods of faecal sample analysis *viz.* modified Ziehl–Neelsen (Henricksen and Pohlenz, 1981), Kinyoun (Soave, 1983), Sucrose floatation (Barwick *et al.,* 2000), immunoflourescence test (Alles *et al.,* 1995), Safranin methylene blue staining (Baxby *et al.,* 1984), Formol-ether concentration (Allen and Ridley, 1970), DMSO carbol-fuchsin stain (Pohjola *et al.,* 1984), Auramine-phenol (Casemore *et al.,* 1984), Salt floatation (Weber *et al.,* 1992), Sucrose centrifugal floatation (Anderson, 1981), Sheather's sugar floatation (Fayer *et al.*1997), Modified Sheather's floatation technique (Current *et al.,*1983*)* antigen-capture ELISA (Newman *et al.,* 1993), for demonstratation of oocysts of *Cryptosporidium* spp. have been cited in the literature. Since it was the first survey of cryptosporidiosis in bovines from Maharashtra, four commonly used methods *viz.* Direct faecal smear examination (DFSS), Normal saline sedimentation (NSS) Sheather's floatation (SF) and Sheather's Floatation Sedimentation smear (SFSS) were employed not only to fulfil the objective but also to compare the efficacy of each method. Further sensitivity of each method was also determined (**Table. 1 & Fig. 1**) in order to know the best method for carrying out large scaled surveys in future. Amongst the four methods employed in the present study, SFSS (Sheather's Floatation Sedimentation smear) was found to be the most sensitive, since 82.98% of total positive cases were detected by this method. The sensitivity in decreasing order of 56.74%, 33.33% and 24.11% was noted for NSSS, SF and DFSS, respectively. Considerably lower sensitivity rate for DFSS and SF noted here could be due to failure of the two technique to detect light infections since in DFSS very little faecal sample was used for preparing direct smear and SF was the only method among the four techniques employed where staining was not done. Thus, it is quite possible

that some of the lightly infected cases could not be detected due to the flaws in the techniques. In contrast in NSS and SFSS, the oocysts were concentrated in one portion of faecal suspension and that portion was used for preparation of smear for staining with modified Ziehl Neelsen method and hence higher percentage of sensitivity could be obtained. The literature in this regards reveals different permutation combinations of concentration medium (formol-ether concentration, Salt floatation, Sucrose centrifugal floatation, Zinc sulphate floatation) and staining agents (Safranin-methylene blue stain, DMSO carbol-fuchsin stain, Kinyoun stain, modified Ziehl Neelsen staining, Auramine-phenol procedures (Allen and Ridley,1970; Anderson, 1981; Henricksen and Pohlenz, 1981; Soave, 1983; Baxby *et al.,* 1984; Pohjola *et al.,* 1984; Casemore *et al.,* 1984 and Weber *et al.* , 1992).

In the present investigation, it was clearly evident that no single method is adequate to detect cent percent cases. Even higher sensitivity of 82.98% by SFSS method indirectly reflects here that 17.02% cases (24) were missed out by this method. The analysis of these 24 samples which were negative by SFSS but shown positive result with one or more of the remaining three methods are depicted in **Table 2**. This analysis was exercised to know whether cent percent sensitivity could be achieved by combining any one method amongst the remaining three with SFSS. However, the outcome of this analysis failed to point out two methods that could achieve 100% sensitivity. Nonetheless by combining SFSS and NSS the sensitivity could be elevated to 92.20% as 13 cases which were negative by SFSS shown positive result with NSS. Since cryptosporidiosis in bovines is usually subclinical or chronic self limiting infection particularly in young calves and immunocompetant individuals, the sensitivity rate detected by SFSS alone and in combination of with NSS in the present study appears to be satisfactory. **Table 2** also reflects that, remaining 11 cases (7.80%) were detected by either SF or DFSS or both the tests which exhibited lower sensitivity than the other two methods. This could be attributed to either rupture of delicate oocysts due to excessive shrinkage in SFSS or technical errors during the procedure or variation in the species of Cryptosporidium that show discrepancy in taking up the stain.

In the present study, for determination of prevalence of cryptosporidiosis in cattle and buffaloes in Mumbai, the findings of all the four the techniques were considered. The oocyts encountered in the present study showed great variation

in size, shape, intensity and staining reaction (**Plate 1**). Majority of the stained oocysts were dark pink in colour with a distinct vacuole or unstained portion or hollow inside. Few oocysts also revealed presence of sporozoites at the periphery. The oocysts were very small. Ten oocysts from each slide were measured by micrometry which ranged from 4µm to 7.4µm. There was no difference in the appearance and measurement of oocsyts encountered in cattle and buffalo samples. Since number of species of Cryptosporidium is known to infect cattle and buffaloes which are indistinguishable on the basis of morphology and micrometry, diagnosis was restricted to generic level. The description of oocysts encountered in the study tallies with morphology of oocysts described by Jaybal and Ray (2005), Roy *et al. (*2006), Rekha *et al.* (2009), Paul *et al*. (2009) and Venu *et al*. (2013). Among different species of Cryptosporidium *viz*. *Cryptosporidium parvum,* (Paul *et al*., 2008) *Cryptosporidium bovis* (Paul *et al.,* 2008), *Cryptosporidium ryanae* (formerly known as Cryptosporidium deer-like genotype (Santin *et al.,* 2004; Fayer *et al.,* 2006 & 2008) and *Cryptosporidium andersoni* (Paul *et al.,* 2009) commonly reported in bovines in different parts of India, the oocysts size of *Cryptosporidium andersoni* is bigger (5.8- 7.2 µ in diameter) than the rest (Upton and Current, 1985; Lindsay *et al.,* 2000 and Fayer *et al.,* 2001) and hence *C. andersoni* can be identified on the basis of micrometry tentatively. In the present study oocysts higher than 5.8 µ in were encountered in 9 samples. Thus tentative prevalence of *C. andersoni* was 2.3%.

4.1.1 Agewise prevalence of cryptosporidiosis in cattle and buffaloes of Mumbai region (Table 3 & Fig.2):

Amongst 391 faecal samples tested for presence of oocysts, 141 were found positive highlighting prevalence rate of 36.06%. The prevalence was slightly higher in buffaloes (38.56%) as compared to that in cattle (34.48%) and the difference was statistically non significant . Similar trend of higher prevalence rate of cryptosporidiosis in buffaloes than cattle was also noted by Prasad *et al.* (1989), Jaybal and Ray (2005) and Bhat *et al*, (2012) . However, Chattopadhyay *et a*l. (2000), Yadav *et al*. (2011) and Venu *et al.* (2012) noted reverse trend. Number of surveys have been conducted in India and comparatively the overall prevalence noted here is in agreement with Paul *et al.* (2008), Maurya *et al.(* 2011), Bhat *et al.* (2012) and Venu *et al.* (2012). In contrast less than 10% prevalence in cattle or buffaloes or in both the species of host was recorded by

Chattopadhyay *et al*. (2000), Mallinath *et al*. (2009), Rana *et al*. (2011), Veena *et al.* (2011) and Rekha *et al.* (2011) in West Bengal, Karnataka, Andhra Pradesh and Hariyana states. Obviously this discrepancy in the prevalence rate noted here and that reported by number of workers across the length and breadth of India is reflection of variations in the managemental practices adopted at different places. Venu *et al*. (2012) reported lower prevalence rate of cryptosporidiosis in bovines maintained at well organised farm as compared to that maintained in small groups by different farmers. It is interesting to note here that, in the study under this publication, except 36 cattle and 49 buffaloes at college livestock instructional farm, all the other faecal samples belonged to the animals from unorganised sector. All the samples from college farm were negative for oocyst of *Cryptosporidium* spp. Apart from college farm no other well organised cattle and buffalo farm was covered in the study and there was large difference in the number of samples from organised farm (college cattle farm) and unorganised sectors and hence the temptation to compare the results as per managemental practices was avoided. The other reason for variation in the prevalence rate could be the method employed for detection of oocysts, sample size and also adeptness of the technicians.

Age predisposition of bovine cryptosporidiosis with higher rate in young calves has now been a well established fact. Infact there are number of references in the literature showing inverse correlation between prevalence rate and advancing age (Santin *et al.,* 2008; Mallinath *et al.,* 2009; Yadav *et al.,* 2011 and Bhat *et al.,* 2012). However, Ondráčková (2009) was isolated in this scenario in reporting reverse trend of higher prevalence rate in adults as compared to calves. Cryptosporidiosis cases at adulthood stage are basically linked with some other primary factors such as immunosupression (Banerjee, 2012), malignancy and from transplant recipients (Ajjampur and kang, 2008). The fact that prevalence rate is higher in calfhood and adulthood cases are invariably secondary to the condition stated above, clearly points out that immunity of host contributes substantially in occurrence of cryptosporidiosis.

In the present study, as expected, prevalence rate of cryptsporidosis was found to be higher in calves below three month of age as compared to adults and the differences were statistically significant at 5% and 1% level in cattle and buffaloes respectively (**Table 3**).However difference in the occurrence rate in

cattle and buffalo calves was statistically non significant. The data on prevalence rate in calfhood in cattle and buffaloes were arranged month-wise in to three groups *viz*.0-1,1-2 and 2-3, and subjected to statiscal analysis using Chi square test which revealed significant difference at 1% level only in buffaloes and in cattle group it was non significant. These findings in the light of available literature reveal self limiting as well as opportunistic nature of the enteric apicomplexan organisms. In the present study, out of 48 adults bovine,only three (6.2%) revealed oocysts of Cryptosporidium in the faecal sample.

4.1.2 Gender wise prevalence of bovine Cryptosporidiosis in Mumbai: (Table 4 & Fig.3)

Prevalence of cryptosporidiosis was found to be marginally higher in male calves as compared to female calves and the differences were statistically non significant in cattle as well as buffaloes. Similar trend of higher prevalence in males as compared to females was also reported by Paul *et al*. (2008), Nouri and Toroghi (1991). However, reverse trend of higher prevalence of cryptosporidiosis in females than in males was recorded by Venu *et al*. (2012) and Bhat *et al*. (2012). From these findings, it appears that there is no gender discrimination in the occurrence of cryptosporidiosis in bovines in India.

4.1.3 Season-wise prevalence of bovine cryptosporidiosis in Mumbai: (Table 5 Fig.4)

Present study was conducted over the period of eight months from November 2012 to June 2013 and thus data pertaining to three distinct season *viz*. winter (November 2012 to February 2013), summer (March to May 2013) and monsoon (June 2013) generated are presented in **Table 5**. It is reflected from the table that, overall prevalence of cryptosporidiosis in bovines in Mumbai was highest in winter (40.5%) followed by that in monsoon (35.63%) and summer (29.41%) and same trend was obtained for cattle as well as buffaloes although all these difference were statistically non significant. Seasonal dynamics in the occurrence of cryptosporidiosis in bovines has been projected by number of authors (Roy *et al.,* 2006; Yadav *et al.,* 2011 & 2012) with wide variation. The variation in the seasonal trend might be attributed to number of climatic factors such as temperature, humidity etc. which influence the viability *vis-a-vis* longevity of free living lifecycle stages (oocysts) and thus has direct bearing on the level of environmental contamination and source of infection. Since the lifecyle of

cryptosporidium is completed within 3 weeks, seasonal factors play very important role in the piling up of infection and increasing its intensity at susceptible age .When data were split into calfhood and adult categories, it was found that in adult the prevalence of cryptosporidiosis was higher in summer followed by that in monsoon and winter. However it has been categorically stated in the literature that occurrence of the disease in adult bovines is usually opportunistic owing to underlined primary predisposing factors such as immunocompression due to physiological stress, prolonged corticosteroid therapy ,concurrent prolonged infection and malignancy (Ajjampur and Kang, 2008) and not linked with season related components.

4.1.4 Prevalence of bovine cryptosporidiosis in relation to faecal consistency: (Table 6 & Fig.5)

Prevalence of cryptosporidiosis was found to be higher in hosts having diarrhoea at the time of collection than those passing faeces of normal consistency although the differences were statistically non significant. Same trend of higher frequency of occurrence of oocysts of *Cryptosporidium* spp. in liquid or watery faeces of cattle and buffaloes has also been published by Kaminjolo *et al.* (1993), Das *et al.* (2003), Roy *et al.* (2006), Singh *et al.* (2006), Paul *et a*l. (2008) and Rekha (2009). Hence the observation of this study endorses the findings of these reports. It also indirectly suggest that at the young age diarrhoea could be caused due to severe infection of Cryptosporidium, in addition to other enteric pathogens viz, *Eimeria zuernii*, *E.bovis*, *E. bareilly* (Maurya *et al*., 2011), calf dirrhoea by colibacillosis and *Toxocara vitulorum* (Soulsby, 1982*).* In the present study, although special efforts were not taken to determine prevalence of other enteric pathogens ,concurrently stages of these pathogens *viz. Eimeria* spp, *Strongyloides papillosus*, *Toxocara vitulorum* were encountered more frequently than not. Hence it is necessary to treat such infections with anticoccidials ,anthelmintics, antibiotics and antidiarrhoeals immediately to control these infections. Simultaneously it will also cut down the source of infection for other individuals in the herd. If these infections in young calves are overlooked and not addressed in time there are chances that such animals, owing to chronic illness during growing phase of life, show stunted growth leading to delay in puberty and below par reproductive performance resulting into economic losses. Adulthood diarrhoea cases should be investigated

for detection of primary cause along with cryptosporidium specific chemotherapy such as Azithromycin (Kadappu, *et al*., 2002).

The epidemiological findings surfaced during current investigations clearly suggest that cryptosporidiosis is common in calves below three month of age in Mumbai region and hence special efforts need to be taken to detect ,treat and control these infections to curb economic losses.

4.2 Molecular identification of *Cryptosporidium* spp.

(Tables 7-10 & Plates 2 - 4)

After scanning through the available literature it was revealed that at least four species of Cryptosporidium *Viz. Cryptosporidium parvum, C. bovis, C. andersoni and C. ryanae (Cryptosporidium* deer like genotype) are known to commonly infect bovines throughout the world (Fayer *et al*,. 2006, Santin *et al*., 2004, Xiao *et al*., 2004 and Banerjee, 2012). These species of Cryptosporidium are almost indistinguishable by conventional methods. Although different species of Cryptosporidium show age related occurrence (Fayer *et al*. 2006, Santin *et al*., 2004) and *C. andersoni* oocysts are bigger than that of other species of Cryptosporidium, the species identification cannot be absolutely relied upon these factors. Thus, pinpoint identification of the species of Cryptosporidium is possible only with molecular tools such as PCR. Species specific diagnosis of Cryptosporidium in a geographical region is necessary since some species have public health significance. The literature search in this regard revealed that in the molecular identification of *Cryptosporidium* spp. different genomic and species specific DNA *viz*. i) SSU rRNA, (Xiao *et al.,*1999) ii)HSP70 (Ryan *et al.,* 2003 & Ng *et al*., 2006). iii) *Cryptosporidium* oocyst wall protein (COWP) (Xiao., *et al*.,2000) and iv) GP60 gene (Alves *et al*., 2003), have been exploited for primary and secondary (nested) amplification (PCR) to demonstrate genus specific band employing electrophoresis of the product. The nested PCR of all the nine samples employed in the study exhibited distinct band of 834 bp on agarose gel (**Plate 2**). Further, the amplicon was subjected to either sequence analysis and or Restriction Fragment Length Polymorphism (RFLP) analysis by employing different enzymes for fragmentation. Thus different restriction enzymes *viz. SspI* (Xiao *et al*.,1999), *VspI* (Xiao *et al.,*1999) and *MboII* (Feng *et al.*, 2007) were

employed for digestion of PCR product to obtain various fragments of different sizes (base pairs) on agarose gel electrophoresis. **Table 7** provides basic information on RFLP analysis of 18s rRNA for differentiation of *Cryptosporidium* spp. occurring in cattle using three commonly employed restriction enzymes (Feng *et al*., 2007).

Table 7: Differentiation of *C. parvum, C. bovis*, *C. andersoni* and Cryptosporidium deer-like genotype by RFLP analysis with *SspI, VspI* and *MboII* (Feng *et al*. 2007)

***Cryptosporidium* Spp.**	**PCR product size (bp)**	***SspI* products (bp)**	***VspI* products (bp)**	***MboII* products (bp)**
C. parvum	**847**	449, 267, 108, 12*, 11*	628, 115, 104	**771**, 76
C. bovis	**835**	432, 267, 103, 33*	616, 115, 104	**412,** 185*, 162, 76
***C. bovis* in yak**	**835**	413,267,103,33*,19*	616,115,104	412,185*,162,76
***C.ryanae* (Deer-like genotype)**	**835**	432, 267, 103, 33*	616, 115, 104	**574**, 185*, 76
C. andersoni	**835**	448, 397	**730**, 115	**769**, 76

*****Not visible in agarose gel electrophoresis

Careful evaluation of RFLP banding pattern on agarose gel by *Ssp*I, *Vsp*I and *Mbo*II depicted in the Table, outwardly poses a complex picture. However, by considering various permutation combinations and focusing only on certain specific bands *viz.*, 730 bp (VspI), 771, 769, 574 and 412 bp (MboII) printed in bold in Table 7, a simplified Table can be formulated for differentiation of commonly encountered species of Cryptosporidium in bovines (**Table 8**).

Table 8: Differentiation of commonly occurring bovine *Cryptosporidium* spp. on the basis of RFLP-PCR results

Restriction Fragment	Restriction Enzymes		Species Identification
	VspI	***MboII***	
771bp	**NA**	✓	***C. parvum***
730bp	✓	**NA**	***C. andersoni***
574bp	**NA**	✓	***C.ryanae***
412bp	**NA**	✓	***C. bovis***

Thus, from the **Table 8,** it is evident that *C. bovis and C. ryanae* can be identified by locating 412 and 574 bp bands of *MboII* RFLP, respectively. *C. andersoni* can be identified by demonstration of 730 bp band of *VspI* RFLP. *C. parvum* can be diagnosed by the combination of (i) absence of 730 bp band of VspI RFLP and (ii) presence of 771 bp band of MboII RFLP. Further 730 bp of *Vsp*I RFLP, 574 bp and 412 bp of *Mbo*II RFLP can be considered as specific bands on electrophorised agarose gel for identification *C. andersoni, C. ryanae* and *C. bovis,* respectively. The comparison also revealed that RFLP with *Vsp*I and MboII is adequate to differentiate species of bovine Cryptosporidiosis and *Ssp*I does not add to the diagnostic value. Venu *et al*. (2012) also categorically concluded that, RFLP analysis of Nested PCR product of 18 s rRNA gene could differentiate two of the commonly occurring *Cryptosporidium* spp. *viz, C. ryanae* and *C. bovis* based of *Mbo*II restriction enzyme. Thus RFLP analysis with VspI and MboII can be put to use for species identification in large scaled epidemiological surveys.

In the present study, six buffaloes and three cattle faecal samples that tested positive by conventional techniques were subjected to molecular analysis

in order to identify the species of Cryptosporidium. All the nine samples had large number of small sized oocysts. The samples after DNA extraction were subjected to primary and secondary (nested) PCR and RFLP analysis of 18S rRNA amplicon was done with all the three restriction enzymes to verify the application of **Table 8.** The results of RFLP analysis are depicted in **Table 9 & Plate 3**. The species of Cryptosporidium were identified by analysing the information of **Table 9** in relation to **Table 8**. All the nine cases revealed infection with single species of Cryptosporidium and no mixed infection was noted. Similar trend of majority of pure infection involving single species of Cryptosporidium was also reported by Paul *et al*. (2008), Venu *et al*. (2012), Khan *et al*. (2010). Out of three faecal samples from cattle, two revealed *C.parvum* and remaining one showed *C.ryanae* infection. Amongst 6 buffalo samples, three were found to be positive for *C.ryanae*, one sample showed *C.parvum* and remaining two did not show splitting of fragments with *MboII.* Thus RFLP analysis of representative bovine faecal samples revealed *C. ryanae* and *C. parvum*. The results of sequence analysis of nested PCR product using NCBI BLAST tool (**Plate 4**) are furnished in **Table 10**. The comparison of RFLP-PCR and sequence analysis results showed good correlation although sequence analysis also showed homology with species of Cryptosporidium other than those detected by RFLP analysis. This could be attributed to close locations of these species particularly *C.ryanae* and *C.bovis* on phylogenetic tree. Similar treand was also reported by Venu *et al*. (2012). Since this is the first study on cryptosporidiosis in bovines in Mumbai and Maharashtra, the main objective of the study was to identify the species of *Cryptosporidium* prevailing in the region. Thus molecular studies including RFLP and sequence analysis clearly indicate presence of three species *viz C. ryanae*, *C. parvum* and *C. bovis* in bovines of Mumbai region. These findings are in general agreement with the observation of Paul *et al*. (2008), Venu *et al*. (2012) and Khan *et al*. (2010) from different regions of India. *Cryptosporidium andersoni,* although not encountered during molecular analysis due to limited number of samples, were tentatively detected in nine faecal samples by micrometric evaluation of the oocysts. Thus when findings of conventional and molecular tools were put together for interpretation, it can be concluded that all four common species of cryptosporidium *viz. C. parvum, C. bovis, C. ryanae* and *C. andersoni* are prevalent in Mumbai.

4.3 Assessment of zoonotic risk of bovine cryptosporidiosis

Cryptosporidium spp. infection in India was first reported in humans in 1985 (Mathan *et al*.,1985) which was followed by number of case reports in children and immunocompromised adults (Das *et al*.,1987; Saraswathi *et al*.,1988; Schgal *et al*., 1989 and Uppal and Natrajan,1991). Correspondingly preliminary work on animal cryptosporidiosis was initiated in 1990 by team of experts on apicomplexan protozoa lead by Dr.J.P. Dubey in north India, during which survey of cryptosporidial infection was carried out in 1990 and 1992 involving cattle and buffalo calves from Bareilly and surrounding areas. The study revealed 17.14% and 10.42% prevalence of cryptosporidiosis (Dubey *et al*., 1992). However species of cryptosporidium was not identified. Detailed review of pertinent Indian literature indicated that although number of studies were parallely conducted in animals and humans , the adequate efforts to establish zoonotic link by cross infection studies were not attempted. Occurance of same Cryptosporium species in animal and human population of the region was put forth by the researchers (Chattopadhyay *et al*., 2000). Among the different species of bovine Cryptosporidium commonly reported from India, only *Cryptosporidium parvum* has been identified as zoonotic spp. In the present study, prevalence of crypytosporidisis in cattle and buffaloes particularly during calfhood was found to be 36.06% and majority of these calves belong to unorganised sectors where hygienic consideration were neglected grossly. Further, molecular analysis few representative samples revealed occurrence of *C. parvum*. In majority of the stables in Mumbai the faeces of the animal accommodated in small temporary sheds are forced with water to the common drain which is occasionally allowed to spread over the land cultivated for green leafy vegetables, thus favouring transmission to human population. The illiterate workers employed at such places staying in the same premises (animal shed) have no knowledge, whatsoever about basics and importance of hygiene leave aside zoonotic significance. Khan *et al*. (2010) showed good correlation of cryptosporidiosis between farm animals and farm workers. Although cryptosporidiosis in immunocompetant individuals are subclinical and self limiting, it takes prolonged pathogenic course in immunocompromised individuals, particularly in HIV/AIDS patients. Opportunistic feature of cryptosporidiosis has been amply demonstrated in the literature though number of reports from Mumbai region (Lanjewar, *et al*., 2013; Chowdhary and Joshi, 2002; Dalvi *et al.*, 2006 and De *et al*., 2009,).Cryptosporidiosis as a disease entity has not received

adequate attention in India owing to number of factors such as ignorance among the medical and veterinary clinicians, preponderance of other overt tropical diesese, small size of organisms for reliable detection etc. Nevertheless cryptosporidiosis in India is emerging as endemic infection due to number of incriminating factors such as cross infectivity among broad range of host species , high biotic potential under tropical climatic conditions, unsatisfactory hygiene, poor sanitary conditions and lack of awareness (Banerjee, 2012). Under these circumstances there exists great risk of zoonotic transmission of cryptosporidiosis from animals to man. Further, report of Rajendran *et al.* (2011) describing occurrence of C*ryptosporidium hominis*, a species confined to humans , in calf in southern India alarmingly indicates synanthropic transmission and possibility of animal species as propagating host, can not be ignored. Thus, there is an urgent need to create awareness about the infections among the veterinarians and medicos so that the information can be percolated in the digestible form to the community coming under professional hazard category such as animal handlers, agriculture workers, sanitary workers, plumbers, hospital workers etc. Improvement in the awareness and overall hygienic and sanitary conditions is the key of comprehensive control of cryptosporidiosis in the years to come.

5. SUMMARY AND CONCLUSION

The present study to note prevalence of cryptosporidiosis in cattle and buffalo population in Mumbai region was conducted from November 2002 to June 2013. Thus a total of 391 faecal samples including 125 from cattle and 218 from buffalo were collected and subjected to four different conventional methods *viz*,Direct faecal smear examination (DFSS), Sheather's floatation method (SF), Normal saline sedimintation smear (NSS) and Sheather's floatation sedimentation smear (SFSS) to demonstrate oocysts of cryptosporidium species. When results of all the four methods were analysed, it was revealed that no single technique is adequate to detect all the positive cases. The comparative efficacy of the four techniques employed in the study, revealed highest sensitivity (82.98%) of SFSS followed by that of NSS (56.74%) , SF (33.33%) and DFSS (24.11%). Further combination of SFSS and NSS test could detect 92.20% cryptosporidiosis cases. Considering results of all the four techniques, the overall prevalence of bovine *cryptosporidiosis* was found to be 36.06% with marginally higher rate of occurrence in buffaloes than in cattle. In both the species of host, the prevalence was distinctly higher in calves below 3 months of age as compared to that in adults. Infact further split of data pertaining to calves into three agewise subgroup *viz*.0-1 month,1-2 months and 2-3 months exhibited highest prevalence in calves below one month of age followed by 1-2 months age group and 2-3 months age group ,irrespective of species of host. In general the differences in prevalence rate of cryptosporidiosis in different age groups were statistically significant.Thus present study showed inverse correlation between the prevalence rate of cryptosporidiosis and age of the host. Higher prevalence of cryptosporidiosis was recorded in winter (40.54%) followed by that in monsoon (35.63%) and summer (29.41%), irrespective of species of host ,though the differences were statistically non significant. Analysis of data pertaining to gender of host and consistency of faecal samples highlighted higher, but not statistically significant, prevalence rate in males (41.67%) as compared to females (31.84%) and in calves with dirrhoea (42.07%) as compared to calves with apparently normal faecal consistency (31.72%). Oocysts of *Cryptosporidium* spp.detected during conventional faecal sample analysis were extremely small ranging from 4.05 ± 0.052 to 7.4±0.835 µm in diameter. The oocysts in stained preparations appeared as dark pink bodies of different shape with a hollow inside. In few

oocysts sporozoites were also seen at periphery. Larger oocysts having diameter more than 5.7±0.835 to 7.2±0.835 µm were tentatively identified as *Cryptosporidium andersoni.*

Nine faecal samples including three from cattle calves and six from buffalo calves that revealed oocysts of *Cryptosporidium* spp. were subjected to molecular analysis by extracting DNA which was subjected to nested PCR for amplification of 18S r RNA gene, revealed 834 bp band on agarose gel. RFLP analysis of PCR product with *SspI, VspI* and *MboII* restriction enzymes identified two species of Cryptosporidium *viz. C. ryanae* and *C. parvum* in seven out of nine samples. In order to confirm the species of Cryptosporidium involved in the infection, the amplicons were sequenced and the analysis revealed three species *viz.C.ryanae ,C.parvum and C.bovis* . Comparison of PCR- RFLP and sequencing results showed excellent correlation. Thus four different species of Cryptosporidium *viz C.ryanae ,C.parvum and C.bovis* (molecular analysis) and *C.andersoni* (micrometric analysis) were found prevalent in calves residing in Mumbai region.Occurance of *Cryptosporidium parvum*,a well known zoonotic apicomplexan opportunistic protozoa in calves in Mumbai region was considered to be a serious public health hazard to associated human population particularly during immunocompramised situation.

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THESIS ABSTRACT

a)	Title of the thesis (in Capital letters)	**:**	PREVALENCE OF *CRYPTOSPORIDIUM* SPP. INFECTION IN CATTLE AND BUFFALO CALVES IN AND AROUND MUMBAI REGION
b)	Full name of student	**:**	Hingole Arati Chandramuni
c)	Name and address of Major Advisor	**:**	Dr. M. L. Gatne Professor Dept. of Veterinary Parasitology, Bombay Veterinary College, Parel
d)	Degree to be awarded	**:**	M. V. Sc.
e)	Year of award of degree	**:**	2013
f)	Major subject	**:**	Veterinary Parasitology
g)	Total number of pages in the thesis	**:**	40
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i)	Signature of Student	**:**	
j)	Signature, Name and address of forwarding authority (HOD / SH)	**:**	Dr. M. L. Gatne Professor Dept. of Veterinary Parasitology, Bombay Veterinary College, Parel
	Signature of the Associate Dean		

ABSTRACT

Faecal samples of cattle and buffaloes residing in Mumbai region were collected from November 2012 to June 2013 and investigated by conventional and molecular tools in ordered to note prevalence of Cryptosporidiosis and species involved in the infection. Conventional analysis involving *viz* Direct Faecal Smear Examination (DFSE), Normal Saline Sedimentation (NSS), Sheather's Floatation (SF) and Sheather's Floatation Sedimentation Smear (SFSS) methods demonstrated oocysts of cryptosporidium in 141 (36.06%) out of 391 faecal samples processed during the period with marginally higher of occurrence in buffaloes (36.99%) as compared to cattle (34.48%). Season wise and gender wise analysis of results revealed higher prevalence in winter (40.54%) as compared to monsoon (35.63%). and summer (29.41%) and in males (41.67%) as compared to females (31.84%)) irrespective of bovine species of a host. Further, diarrhoeic loose faeces showed higher rate (42.07%) of occurrence of oocysts than apparently normal faecal samples (31.72%) irrespective of the species of the host. All these difference were statistically non significant, However when data were arranged according to age wise groups *viz* calves of 0-1 month, 1-2 months , 2-3 months and adult subjects, the highest prevalence noted in the youngest group (47.12%) declined gradually with the advancing age and it was lowest (6.25%) in adult bovines indicating inverse correlation between prevalence rate of cryptosporidiosis and age of the host. These differences in buffaloes were statistically significant. *Cryptosporidium andersoni* was tentatively identified by morphometric analysis. Molecular approach employing RFLP-PCR and sequence analysis of few samples showed good correlation in the identification of species of Cryptosporidium involved in the infection. Molecular analysis demonstrated occurrence of *Cryptosporidium parvum, C. ryanae, C. bovis.* Thus all the four commonly occurring bovine species of Cryptosporidium were encountered in the study. Public health significance of *Cryptosporidium parvum* encountered in the survey was discussed in the light of epidemiological factors pertaining to the region.

प्रबंध सारांश

1. प्रबंधाचे शिर्षक	:	**मुंबई विभागातील गाई आणि म्हशींमध्ये क्रिप्टोस्पोरिडिओसीस या रोगाच्या प्रादुर्भावाची पारंपारिक आणि रेणुकीय पध्दतीने तपासणी**
2. विद्यार्थ्याचे नाव	:	हिंगोले आरती चंद्रमुनी
3. मार्गदर्शकाचे नाव	:	डॉ. एम.एल. गटणे प्राध्यापक, परजीवीशास्त्र विभाग, मुंबई पशुवैद्यक महाविद्यालय परळ, मुंबई—400 012
4. प्रदान करण्यात येणारी पदवी	:	एम.व्ही.एस.सी.
5. पदवी प्रदान करण्याचे वर्ष	:	2013
6. प्रमुख विषय	:	परजीवीशास्त्र
7. प्रबंधातील एकूण पृष्ठ संख्या	:	40
8. सारांशातील एकूण शब्द संख्या	:	198
9. विद्यार्थ्याची सही	:	
10. प्रबंध पाठविण्या—या अधिका—याचे संपूर्ण नाव, पत्ता आणि सही	:	डॉ. एम.एल. गटणे मुंबई पशुवैद्यक महाविद्यालय, परळ, मुंबई—400 012
11. सहयोगी अधिष्ठाता, मुंबई पशुवैद्यकीय महाविद्यालय, परळ, मुंबई—12	:	

सारांश

मुंबई विभागातील गाई आणि म्हशींमध्ये क्रिप्टोस्पोरिडिओसीस या रोगाच्या प्रादुर्भावाची पारंपारिक आणि रेणुकीय पध्दतीने तपासणी

मुंबई विभागातील गाय वर्ग आणि म्हैस वर्गातील जनावरांच्या शेणांचे नमुने नोव्हेंबर 2012 ते जून 2013 या कालावधीमध्ये गोळा करून पारंपारिक आणि रेणुकीय पध्दतीच्या चाचण्यांव्दारे क्रिप्टोस्पोरिडिओसीस या रोगाच्या प्रादुर्भावाची नोंद करण्यात आली. आणि या रोगाच्या संसर्गाला कारणीभूत असणा–या विविध प्रजातींची देखील नोंद घेण्यात आली. पारंपारिक पध्दतीमध्ये डायरेक्ट फिकल स्मेअर, नॉर्मल सलाईन सेडिमेंटेशन, शिदर्स फलोटेशन, सेडिमेंटेशन स्मेअर या पध्दतीव्दारे 391 शेणांच्या नमुन्यांचे निरिक्षण करण्यात आले, त्यापैकी 141 (36.06%) शेणाच्या नमुन्यांमध्ये क्रिप्टोस्पोरिडिअम प्रजातीचे परजीवी आढळून आले.तुलनात्मकदृष्टया म्हैस वर्ग प्राण्यांमध्ये प्रादुर्भाव(36.99%), गाय वर्ग (34.48%) प्राण्यांपेक्षा थोडा जास्त दिसून आला. मिळालेल्या निकालाचे ऋतूमानानुसार आणि लिंगभेदानुसार पृथ्थकरण केले असता, हिवाळयामध्ये (40.54%), पावसाळयामध्ये (35.63%) आणि उन्हाळयापेक्षा (29.41%) अधिक तसेच नरांमध्ये आला. हे सगळे फरक सांख्यिकीदृष्टया लक्षणीय नाहीत पण मिळालेल्या माहितीची 0–1 महिने, 1–2 महिने, 2–3 महिन्यांची वासरे आणि प्रौढ जनावरे अशी वयोमानानुसार रचना केल्यावर असे आढळून आले की सर्वात कमी वयाच्या वासरांमध्ये प्रादुर्भाव (47.12%) सर्वात जास्त आहे. आणि वाढत्या वयोमानानुसार प्रादुर्भाव कमी होत गेल्याचे दिसून आले. सर्वात कमी प्रादुर्भाव प्रौढ जनावरांमध्ये (6. 25%) आढळून आले, यावरून असे निदर्शनास येते की, जनावरांच्या वाढत्या वयोमानानुसार क्रिप्टोस्पोरिडिओसीसचा प्रादुर्भाव कमी होत जातो. हा फरक गाय वर्ग आणि म्हैस वर्ग या दोन्ही वर्गांमध्ये लक्षणीय आहे. क्रिप्टोस्पोरिडिअम ॲंडरसनी ही प्रजाती, परजीवींच्या पेशीय रचनेच्या अभ्यासाव्दारे ढोबळ मानाने ओळखता आली. रेणुकीय पध्दतीमध्ये, आर.एफ.एल.पी.–पी.सी.आर. आणि सिक्वेन्स पृथ्थकरण याव्दारे काही नमुन्यांचा अभ्यास कोणत्या क्रिप्टोस्पोरिडिअम प्रजाती या प्रादुर्भावामध्ये कारणीभूत आहेत हे ओळखता आले, तसेच या दोन्ही पध्दतींचा एकमेकांशी ताळमेळ आहे हे निदर्शनास आले.रेणुकिय पृथ्थकरणाव्दारे क्रिप्टोस्पोरिडिअम पारवम, क्रिप्टोस्पोरिडिअम रायने आणि क्रिप्टोस्पोरिडिअम बोविस या प्रजाती या भागामध्ये आढळतात हे निदर्शनास आले. अशा रितीने क्रिप्टोस्पोरिडिअमच्या गुरांमधील सर्व चार प्रमुख प्रजातींची या अभ्यासामध्ये नोंद करण्यात आली, तसेच या भागातील एपिडेमिओलॉजिकल घटक लक्षात घेऊन, क्रिप्टोस्पोरिडिअम पारवम या अभ्यासाअंती आढळलेल्या प्रजातींची माणसांच्या सार्वजनिक स्वास्थ्यावरील परिणामांवर चर्चा केली गेली आहे.

VITA

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Later she joined Veterinary Profession at “College Of Veterinary and Animal Sciences” Parbhani in the year 2006 and completed her graduation obtaining the degree B V Sc & A H in the year 2011 securing first class, to pursue higher and specialized degree she joined the studies in the discipline of Veterinary Parasitology, Bombay Veterinary College, Mumbai for her post graduation,

Table 1: Sensitivity of different conventional methods in diagnosis of bovine Cryptosporidiosis

Category of Host	No. of animals examined	Total positive	Samples detected positive by various methods			
			DFSE	SF	NSSS	SFSS
Cattle calves	125	48	13	19	29	42
Buffalo calves	218	90	21	28	51	72
Adult cattle	20	2	0	0	0	2
Adult buffalo	28	1	0	0	0	1
Total	391	141	34	47	80	117
Sensitivity			**24.11%**	**33.33%**	**56.74%**	**82.98 %**

Fig. 1: Sensitivity of different conventional methods in diagnosis of bovine Cryptosporidiosis

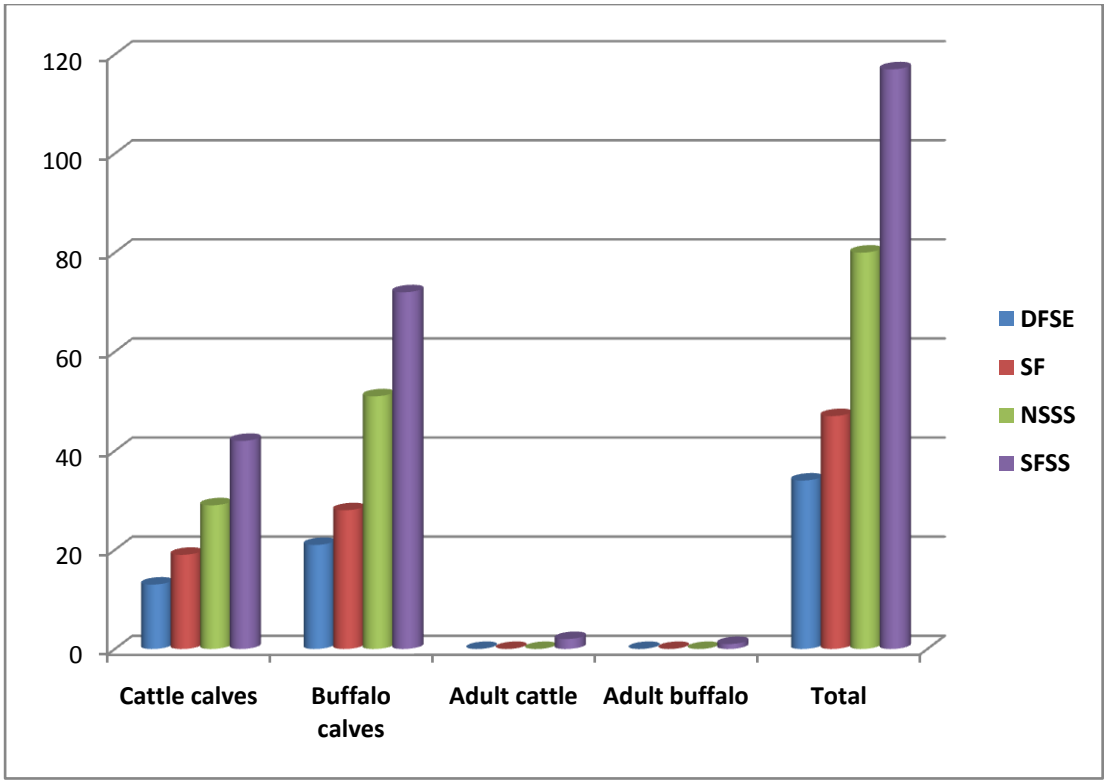


Table 2: Details of bovine samples which revealed oocysts by the methods other than SFSS

Sr. no.	Sample no.	DFSS	SF	NSSS
1	CR21		✓	
2	CR08		✓	
3	PL28	✓		
4	Farm03		✓	
5	Farm07			✓
6	GP19			✓
7	CR11	✓		
8	GP23			✓
9	NR19		✓	
10	NR07			✓
11	PL09		✓	
12	Farm04	✓		
13	Farm 16			✓
14	PL13			✓
15	PL32			✓
16	Farm05		✓	
17	Farm17	✓		
18	GP36			✓
19	Nik 14	✓		
20	319		✓	
21	2001		✓	
22	CVC08			✓
23	531			✓
24	720			✓

Note: Serial no. 1-18 are samples from buffaloes and 19-24 are from cattle.

Table 3: Age-wise prevalence of Cryptosporidiosis in cattle and buffaloes

Category of Host	Cattle calves			Buffalo calves			
Age (months)	No. of animals	No. of positive cases	Positive %	No. of animals	No. of positive cases	Positive %	Total prevalence (%)
0 - 1	62	28	45.16	112	54	48.21	47.12
1 - 2	36	13	36.11	65	26	40	38.61
2 - 3	27	7	25.92	41	10	24.39	25
Total	**125**	**48**	**38.40**	**218**	**90**	**41.28**	**40.23**
Adult	**Adult cattle**			**Adult buffalo**			
	No. of animals	**No. of positive cases**	**Positive %**	**No. of animals**	**No. of positive cases**	**Positive %**	**Total prevalence (%)**
	20	2	10	28	1	3.57	6.25
Grand Total	**145**	**50**	**34.48**	**246**	**91**	**36.99**	**36.06 (141/391)**

A) Statistical significance between age groups in cow calves and adult cattle

Parameter	Total examined	Total positive	Chi-square value	Chi-square Table 5%	Chi-square Table 1%	Significance/ Non significance
Cow calves	125	48	4.02	3.84	5.02	Significant at 5% level of probability
Adult cattle	20	2				
Total	145	50				

B) Statistical significance between age groups in cow calves

Parameter	Total examined	Total positive	Chi-square value	Chi-square Table 5%	Chi-square Table 1%	Significance/ Non significance
0-1	62	28	1.877	5.99	7.38	Non significance
1-2	36	13				
2-3	27	7				
Total	125	48				

C) Statistical significance between age groups in buffalo calves and adult buffaloes

Parameter	Total examined	Total positive	Chi-square value	Chi-square Table 5%	Chi-square Table 1%	Significance/ Non significance
Buffalo calves	218	90	5.499	3.84	5.02	Significant at 1% level of probability (HS)
Adult buffaloes	18	1				
Total	236	91				

D) Statistical significance between age groups in buffalo calves

Parameter	Total examined	Total positive	Chi-square value	Chi-square Table 5%	Chi-square Table 1%	Significance/Non significance
0-1	112	54	6.39	5.99	7.38	Significant at 1% level of probability (HS)
1-2	65	26				
2-3	41	10				
Total	218	90				

E) Statistical significance between cattle calves and buffalo calves

Parameter	Total examined	Total positive	Chi-square value	Chi-square Table 5%	Chi-square Table 1%	Significance
Cow calves	125	48	0.20	3.84	5.02	NS
Buffalo calves	218	90				
Total	343	138				

Table 4: Gender wise Prevalence of cryptosporidiosis in cattle and buffaloes

Category of Host	Male		Female		Total	
	Animals examined	Positive	Animals examined	Positive	Animals examined	Positive
Cattle calves	70	28(40.00)	55	20(36.36)	125	48(38.40)
Adult cattle	Nil	Nil	20	02(10.00)	20	02(10.00)
Cattle total	**70**	**28(40.00)**	**75**	**22(29.33)**	**145**	**50(34.48)**
Buffalo calves	98	42(42.86)	120	48(40.00)	218	90(41.28)
Adult buffalo	Nil	Nil	28	01(03.57)	28	01(03.57)
Buffalo total	**98**	**42(42.86)**	**148**	**49(33.11)**	**246**	**91(36.99)**
Total calves	**168**	**70(41.67)**	**175**	**68(38.86)**	**343**	**138(40.23)**
Total adults	**Nil**	**Nil**	**48**	**03(6.25)**	**48**	**03(6.25)**
Grand total	**168**	**70(41.67)**	**223**	**71(31.84)**	**391**	**141(36.06)**

***Figures in parenthesis indicate percentage**

A. Statistical significance between male and female cattle calves

Parameter	Total examined	Total positive	Chi-square value	Chi-square Table 5%	Chi-square Table 1%	Significance/Non significance
Male	70	27	0.00	3.84	5.02	**Non significance**
Female	55	21				
Total	125	48				

B. Statistical significance between male and female buffalo calves

Parameter	Total examined	Total positive	Chi-square value	Chi-square Table 5%	Chi-square Table 1%	Significance/ Non significance
Male	98	42	0.10	3.84	5.02	NS
Female	120	48				
Total	218	90				

Table 5: Season-wise prevalence of Cryptosporidiosis in cattle and buffaloes

Category of Host	Winter		Summer		Monsoon		Total	
	No. of animals	Positive %	No. of animals	Positive %	No. of animal	Positive %	No. of animal	Positive%
Cattle calves	63	27 (42.86)	40	12(30.00)	22	9(40.91)	125	48(38.40)
Adult cattle	08	01 (12.5)	05	Nil	07	1(14.29)	20	02(10.00)
Cattle total	**71**	**28 (39.44)**	**45**	**12(26.67)**	**29**	**110(34.48)**	**145**	**50(34.48)**
Buffalo calves	103	47 (45.63)	67	22(32.84)	48	21(43.75)	218	90(41.28)
Adult buffalo	11	Nil	07	01(14.29)	10	Nil	28	01(03.57)
Buffalo total	**114**	**47 (41.22)**	**74**	**23(31.08)**	**58**	**21(36.21)**	**246**	**91(36.99)**
Total bovine calves	**166**	**74 (44..58)**	**107**	**34(31.78)**	**70**	**30(42.81)**	**343**	**138(40.23)**
Total bovine adult	**19**	**01 (5.26)**	**12**	**01(08.33)**	**17**	**01(05.88)**	**48**	**03(06.25)**
Grand total	**185**	**75 (40.54)**	**119**	**35(29.41)**	**87**	**31(35.63)**	**391**	**141(36.06)**

A. Statistical significance between the seasons in cow calves

Parameter	Total examined	Total positive	Chi-square value	Chi-square Table 5%	Chi-square Table 1%	Significance
Winter	63	27	1.096	5.99	7.38	NS
Summer	40	12				
Rainy	22	9				
Total	125	48				

B. Statistical significance between the seasons in buffalo calves

Parameter	Total examined	Total positive	Chi-square value	Chi-square Table 5%	Chi-square Table 1%	Significance
Winter	103	47	1.69	5.99	7.38	NS
Summer	67	22				
Rainy	48	21				
Total	218	90				

Table 6: Prevalence of Cryptosporidiosis in relation to faecal consistency

Host	Diarrhoeic faeces		Non diarrhoeic		Total	
	animals examined	Positive	animals examined	Positive	animals examined	Positive
Cattle calves	70	29 (41.43)	55	19(34.45)	125	48(38.40)
Adult cattle	04	01(25.00)	16	01(06.25)	20	02(10.00)
Cattle total	**74**	**30(40.54)**	**71**	**20(28.17)**	**145**	**50(34.48)**
Buffaloe calves	82	39(47.56)	136	51(37.50)	218	90(41.28)
Adult buffaloe	08	Nil	20	01(05.00)	28	01(03.57)
Buffalo total	**90**	**39(43.33)**	**156**	**52(33.33)**	**246**	**91(36.99)**
Total bovine calves	**152**	**68(44.74)**	**191**	**70(36.65)**	**343**	**138(40.23)**
Total bovine adult	**12**	**01(12.50)**	**36**	**02(05.56)**	**48**	**03(06.25)**
Grand total	**164**	**69(42.07)**	**227**	**72(31.72)**	**391**	**141(36.06)**

***Figures in parenthesis indicate percentage**

A) Statistical significance between diarrhoeic and non-diarrhoeic cow calves.

Parameter	Total examined	Total positive	Chi-square value	Chi-square Table 5%	Chi-square Table 1%	Significance
Diarrhoeic	70	29	0.334	3.84	5.02	NS
Non-diarrhoeic	55	19				
Total	125	48				

B) **Statistical significance of diarrhoeic and non-diarrhoeic buffalo calves**

Parameter	Total examined	Total positive	Chi-square calculated	Chi-square Table 5%	Chi-square Table 1%	Significance
Diarrhoeic	82	39	1.56	3.84	5.02	NS
Non-diarrhoeic	136	51				
Total	218	90				

Table 9: Results of RFLP- PCR with *SspI, VspI* and *MboII* restriction enzymes

Sr. no.	Result	Name of Restriction Enzymes		
		SspI	***VspI***	***MboII***
1	*C.parvum*	449,267,108	628,115,104	**774,**76
2	*C.ryanae*	432,267,103,33	616,115,104	**574**,185,76
3	*C.ryanae*	432,267,103,33	616,115,104	**574**,185,76
4	*C.ryanae*	432,267,103,33	616,115,104	**574,**185,76
5*****	*C.parvum*	449,267,108	628,115,104	**774**,76
6*****	*C.parvum*	449,267,108	628,115,104	**774**,76
7*****	*C.ryanae*	432,267,103,33	616,115,104	**574**,185,76

*** samples from cattle**

Table. 10. Results of sequence analysis of bovine Cryptosporidiosis spp.

Sr. No.	Sample no.	Name of species	Homology	Gene Bank accession no
1	CRPM1	*C. parvum isolate* *C. parvum gene*	99% 99%	KC569976.1 AB513881.1
2	CRPM2	*C.bovis* isolate *C.bovis* gene	99% 99%	HQ179573.1 AB746197.1
3	CRPM3	*C.ryanae* gene	99%	AB746196.1
4	CRPM4	*C.ryanae* isolate *C.ryanae* gene	99% 99%	JN400880.1 AB746196.1
5	CRPM5	*C.ryanae* *C.ryanae* isolate *C. ryanae* gene	99% 100% 100%	JN400880.1 JX559847.1 AB712387.1
6*	CRPM6	*C. parvum* isolate *C.parvum* frm IRAN	99% 99%	JX298603.1 HQ651731.1
7*	CRPM7	*C. parvum* strain *C. parvum* gene	83% 83%	KC662502.1 AB746195.1
8*	CRPM8	*C.ryanae* isolate *C.ryanae* gene	99% 88%	JX237831.1 AB712388.1
9	CRPM9	*C.bovis* isolate *C.bovis* gene	99% 98%	JX416364.1 AB441689.1

***Samples from Cattle and Buffaloes**

Fig.2: Agewise prevalence of cryptosporidiosis in cattle and buffaloes

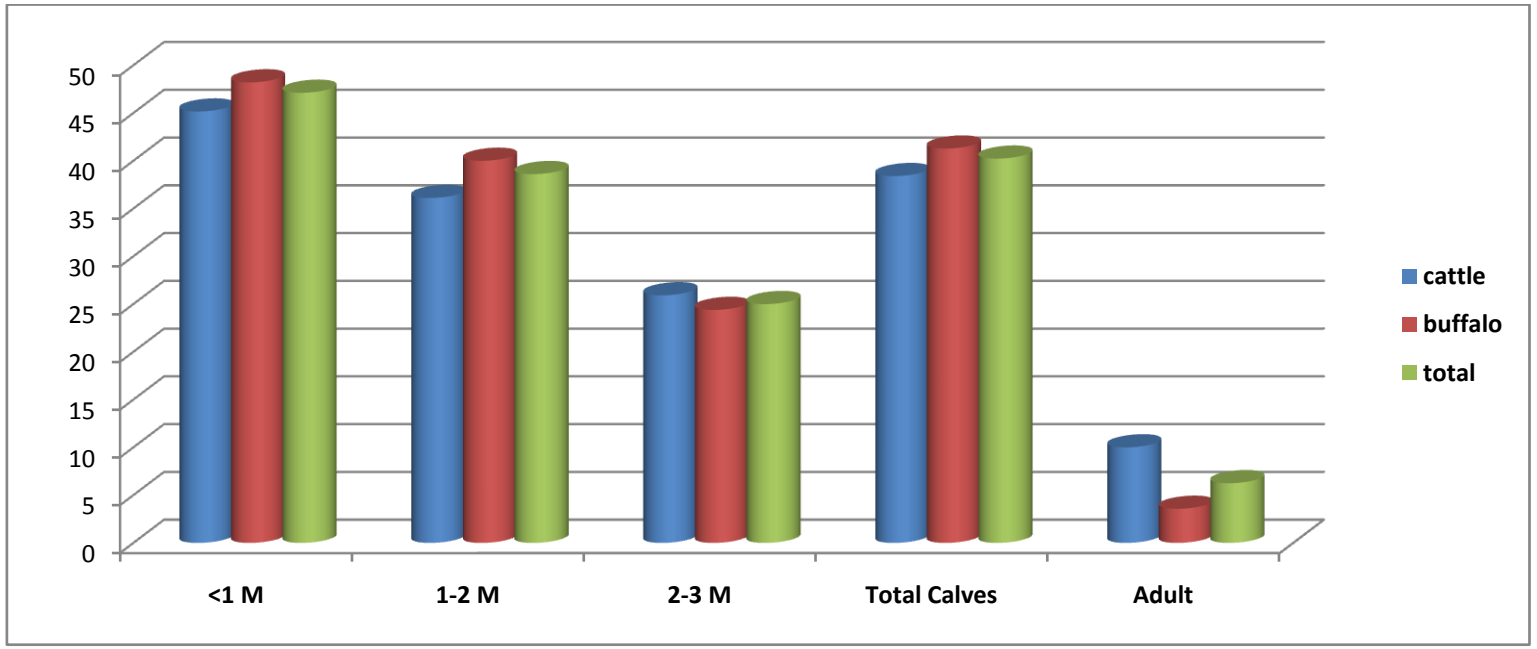


Fig. 3: Gender wise Prevalence of cryptosporidiosis in cattle and buffaloes

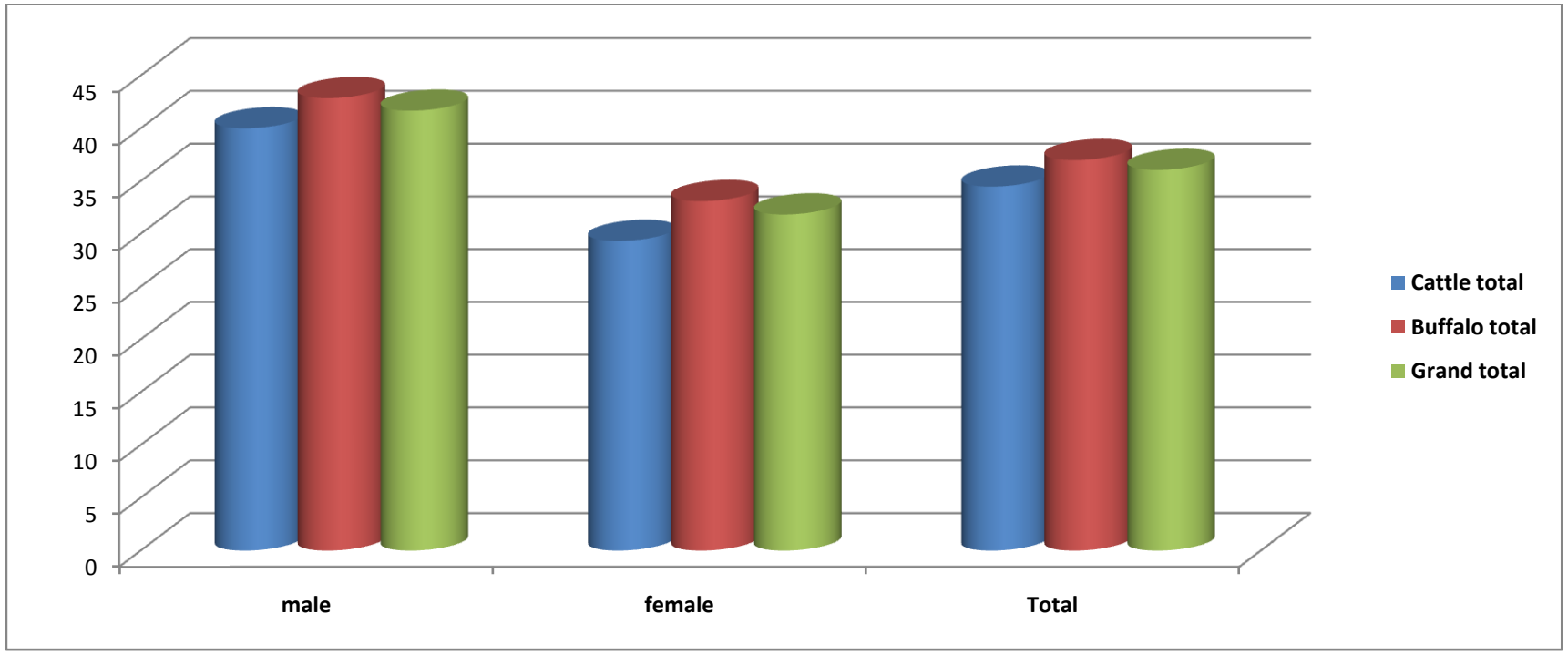


Fig. 4: Season-wise prevalence of Cryptosporidiosis in cattle and buffaloes

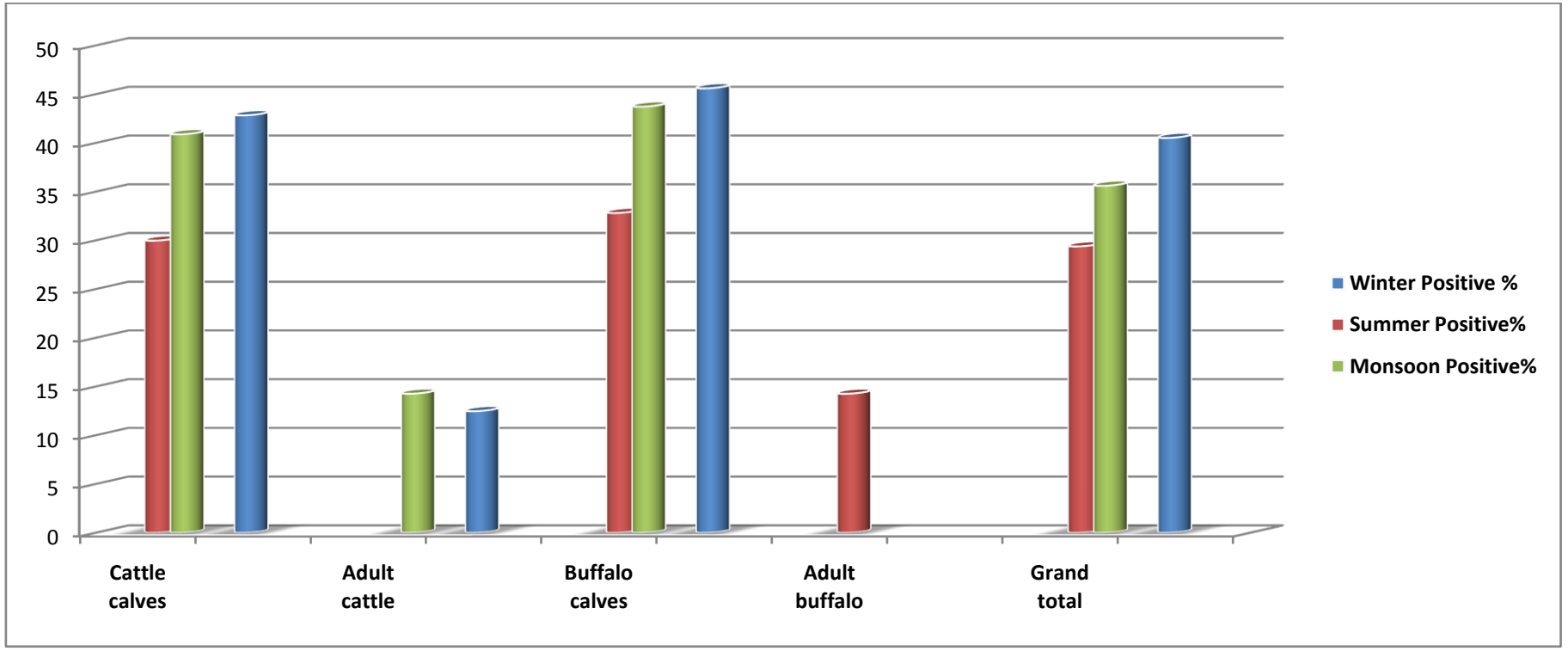


Fig.5: Prevalence of Cryptosporidiosis in cattle and buffaloes in relation to faecal consistency

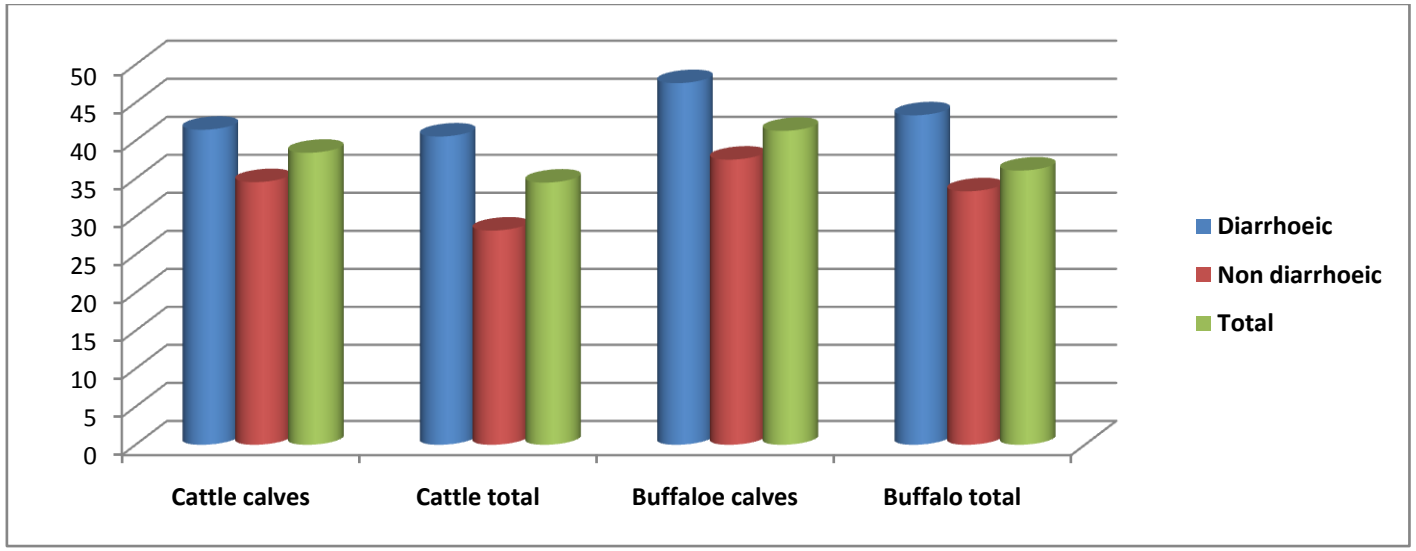
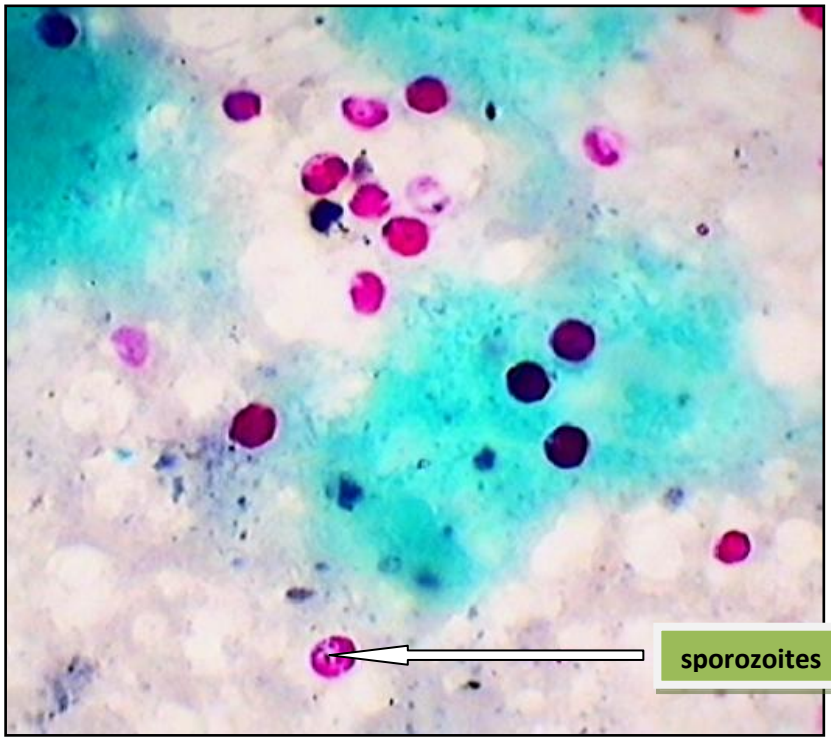
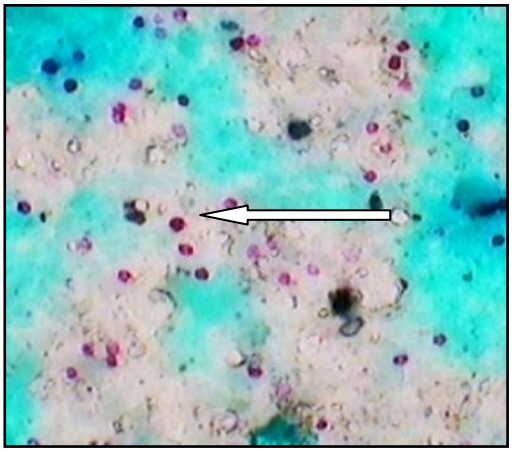


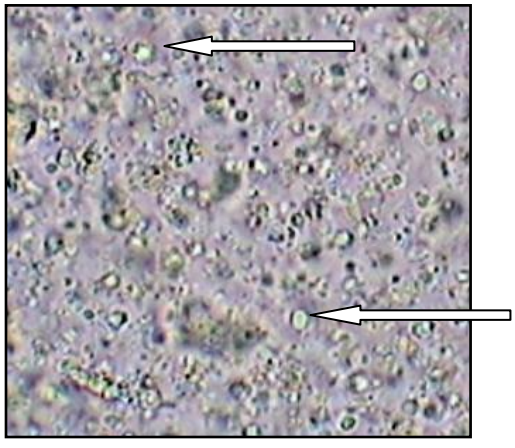
Plate 1. Microscopic images of faecal smear showing oocysts of *Cryptosporidium* spp.



mZN stained oocysts: (100X)

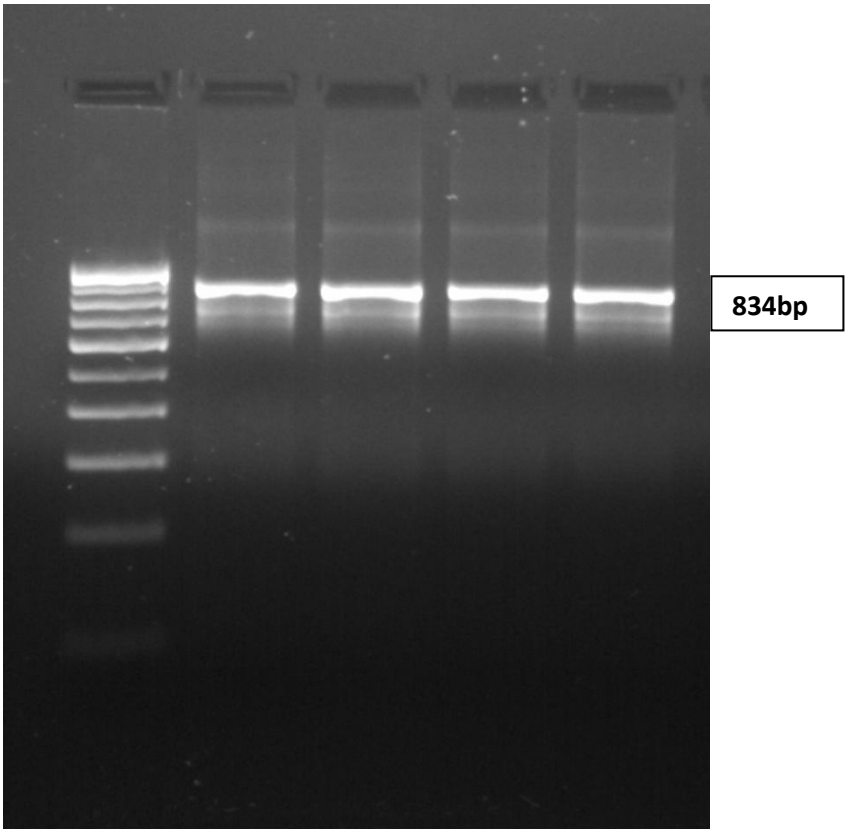


mZN Stained oocysts: (45 X)



Unstained oocysts: (45 X)

Plate 2. Nested PCR Product of Cryptosporidium spp on agarose gel showing 834bp band



Lane 1: 100 bp ladder
Lane 2- 5: Positive samples

Plate 3. Agarose gel showing specific banding pattern of RFLP- PCR of *MboII*

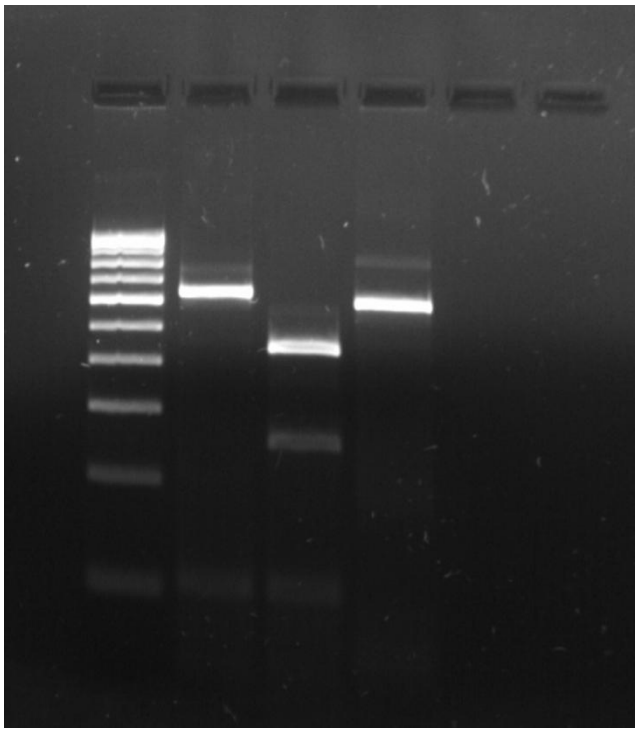
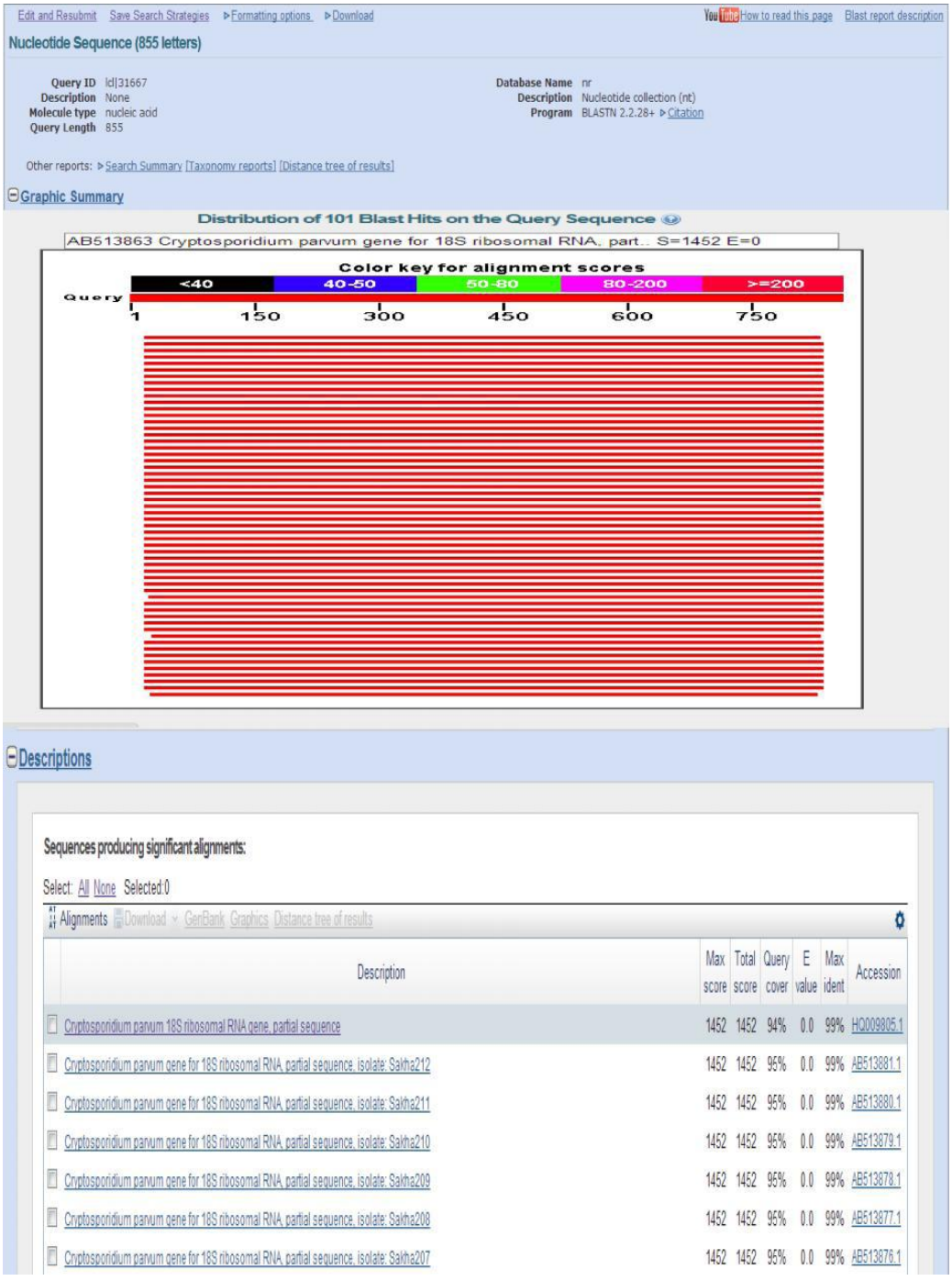


Plate 4. BLAST Analysis of Sequence of Positive PCR Product



Edit and Resubmit | Save Search Strategies | ▹Formatting options | ▹Download | How to read this page | Blast report description

Nucleotide Sequence (855 letters)

Query ID	lcl|31667	**Database Name**	nr
Description	None	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.2.28+ ▹Citation
Query Length	855		

Other reports: ▹Search Summary [Taxonomy reports] [Distance tree of results]

Graphic Summary

Distribution of 101 Blast Hits on the Query Sequence

AB513863 Cryptosporidium parvum gene for 18S ribosomal RNA, part... S=1452 E=0

Color key for alignment scores

<40 | 40-50 | 50-80 | 80-200 | >=200

Query 1 150 300 450 600 750

Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Cryptosporidium parvum 18S ribosomal RNA gene, partial sequence	1452	1452	94%	0.0	99%	HQ009805.1
Cryptosporidium parvum gene for 18S ribosomal RNA, partial sequence, isolate: Sakhs212	1452	1452	95%	0.0	99%	AB513881.1
Cryptosporidium parvum gene for 18S ribosomal RNA, partial sequence, isolate: Sakhs211	1452	1452	95%	0.0	99%	AB513880.1
Cryptosporidium parvum gene for 18S ribosomal RNA, partial sequence, isolate: Sakhs210	1452	1452	95%	0.0	99%	AB513879.1
Cryptosporidium parvum gene for 18S ribosomal RNA, partial sequence, isolate: Sakhs209	1452	1452	95%	0.0	99%	AB513878.1
Cryptosporidium parvum gene for 18S ribosomal RNA, partial sequence, isolate: Sakhs208	1452	1452	95%	0.0	99%	AB513877.1
Cryptosporidium parvum gene for 18S ribosomal RNA, partial sequence, isolate: Sakhs207	1452	1452	95%	0.0	99%	AB513876.1