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Epidemiological Studies and Molecular Diagnosis of *Giardiasis* in Bovine

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Abstract

Epidemiological studies were undertaken at Military dairy farm, Government dairy Farm, Gawala dairy colonies and house hold dairy in Lahore under different managemental and climatic conditions of four different areas at Lahore, Pakistan. Infection rate among the calves, cattle and buffaloes were (50.27%), (28.05 %), and (26.11 %) respectively. In calves, highest prevalence was recorded in Government. dairy farm (68.33%), and the lowest (34.44%) were recorded in household dairy. Overall highest (61.6%) season wise prevalence was recorded during autumn and the lowest (34.1%) was recorded during winter. In cattle, the highest (41.67%) prevalence was recorded at Government dairy farm and the lowest (15%) was recorded in household dairies. Similarly in buffaloes, the highest (40.55 %.) prevalence was recorded at Government dairy Farm and the lowest prevalence (12.77%) was reported in house hold dairies. The non significant (P>0.05) difference was recorded during the analysis of data. It was observed that the higher infection rate was recorded in younger than older animals. Female were more commonly infected than Male.

Key words: Epidemiology, *Giardiasis*, Bovine.

Introduction

Giardia lamblia, also called *Giardia duodenalis* or *Giardia intestinalis*, is a protozoan parasite, which inhabits small intestine and causes extensive morbidity worldwide. *Giardia* was seen under microscope first time by Antony Van Leeuwenhock during 1681 (Dobel, 1920). Later on it was described by Vilim Lambli in 1859. However, most of the research workers in this field use the name *Giardia* (Meyer, 1990) and extensive work has been undertaken on its epidemiology, pathophysiology and giardiasis had been reported in Europe and United treatment (Meyer, 1994). Outbreaks of waterborne States during years 1960s and 1970s (Craun, 1990; Farthing, 1992). The clinical symptoms of the giardiasis in human are diarrhoea, dehydration, abdominal pain, nausea, vomiting and weight loss (Thompson and Monis, 2004). *G. duodenalis* infection is depending on host, parasite and environment (Thompson *et al.,* 1990).

G. duodenalis is the only species observed both in human beings and animals including dogs, cats, bovines, pigs, sheep and equines (Thomson *et al.,* 2000; Thomson 2004). Infection spreads through ingestion of *Giardia* cysts excreted in faeces of infected animals (Monis and Thomson, 2003). *Giardia* is cosmopolitan in distribution and its prevalence has been reported in young cattle and less known in the adult cattle (Xiao and Herd, 1994; Olson *et al*., 1997). *Giardia* infection occurs in group housed calves as well as housed within individual pens (Olson *et al.,* 1997a; Handley *et al.*, 1999). There are some reports on low *Giardia* infection rates (Oviedo *et al.,* 1987) and others as major cause of diarrhoea in calves (Deshpande and Shastri, 1981). Other enterpathogens have not been tested with Giardia *infection* in calf (Xiao *et al.,* 1994; Ruest *et al.,* 1995). *Giardiasis* is associated with infection by particular genotypes in animal and humans as well (*Nash et al.,* 1987). Infected livestock can excrete over 10^6 *Giardia* cysts per gram of faeces, which may contaminate the environment and can act as potential reservoir for human outbreaks of Giardiasis (Buret *et al.* 1990; Handley 2000b).

Diagnosis of *Giardia* by conventional microscopic methods following faecal concentration techniques, particularly zinc sulphate and zinc chloride floatation and centrifugation (Zajac *et al*., 2002) remains a reliable indicator of infection status. However, there is a need for a sensitive and specific diagnostic method to detect etiological agents of disease. For *Giardia*, molecular technique such as polymerase chain reaction (PCR) is an alternate method for specific detection of pathogens in stool. Sensitivity of detection by PCR is greater than that compared with microscopic evidence (McGlade *et al*., 2003).

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Giardiasis is one of the major zoonotic threats in Pakistan. So for, no study has been undertaken to pin point zoonotic potential and molecular diagnosis of Giardiasis in humans and animals. In view high prevalence of Giardiasis in bovine a study was designed to study the epidemiology of *Giardiasis* in bovines to compare its prevalence among buffaloes, cows and calves, and to develop a PCR based technique for diagnosis/detection of *Giardia* infection in animals.

Materials and Methods

Animals examined

A total of 2160 buffaloes, cattle and calves of various ages and of both sexes at government dairy farm, military dairy farm, gawalla colonies and house hold dairies at Lahore were randomly selected and examined for the prevalence of *Giardiasis* during August, 2007 to July, 2008.These animals were randomly divided into three subgroups *i.e*. A, B and C., in group A there were buffaloes, group B contained cows while in group C there were calves.

Sources & collection of specimen

The feces were collected from each animals directly from the rectum using disposables gloves in sterilized plastic bags were used for each animals and these were labelled with information's regarding animal No, date of collection and name of the farm. Attempt was made to collect fresh faeces or freshly passed faeces from the soil. After collection these samples were stored in cooler containing crushed ice and immediately transported to the postgraduate laboratories department of Parasitology, university of veterinary and animal sciences, Lahore.

Isolation of cyst

Giardia cysts were isolated from the fecal samples as the method described by Handley *et al.,* 1999. The procedure was modified according to the buffaloes, cows and calves to accommodate the fecal size of 20 grams. The fecal samples were mixed in 35 ml of phosphate buffered saline in Petri dish, these were thoroughly mixed and the slurry was filtered three times through cheesecloth and poured them into the 50ml centrifuge tube and layer over 15ml of sucrose (specific gravity 1.13). The samples were centrifuged at 800 x g for 5 minutes. The upper layer and the interface layer were transferred through the pipette into other clean centrifuge tube and add the equal amount of phosphate buffered saline and re-centrifuged at 800 x g for 5 minutes. The supernatant was discarded and the pellet was resuspended with PBS to a final volume of 1ml in clean ependorff tube.

Cyst examinations and counting

The resuspended pellet (20 ul) was taken and placed on slide, made a smear and dried the slide at 37ºC .The slides were stained with iron Haematoxylin stain at standard procedure. One slide was examined for each sample. The slides were examined at 10x, 40x and 100x .The slides were compared with positive slide of *G. duodenalis* and were identified on the basis of size and morphology (Lindsay *et al*., 2000). The number of cysts per gram of faeces was calculated using the formula adopted by Handley *et al.,* 1999.

DNA Extraction

The resuspended fecal samples were subjected to DNA extraction by using the tissue DNA extraction kit (GFC) vivantis throughout the experiment. The ependorff tubes were properly numbered and added the 200ul TL buffer, 5 ul proteinase K and 200ul cell lysis enhancer from the tissue DNA extraction kit and incubate and added the 200ul TB buffer mixed thoroughly and placed in water bath at 65ºc for 20 minutes and centrifuged at 13200 rpm for 30 minutes and the supernatant was added the 200ul absolute ethanol mixed immediately and centrifuged at 13200 rpm for 35 minutes. The pellet was resuspended in 750ul washed buffer and centrifuged at 13200rpm for 30 minutes. The pellet was washed 2-3 times until cleared pellet of DNA was obtained. The pellet was added in TBE buffer and stored at -80 ºC.

Amplification of DNA (PCR) and detection

A primers- A 753 bp product of *Giardia* primers was amplified. The forward sequence of primers was G-753 (5-AAGCCGACGACCTCACCCGCAGTGC-3) and synth ID No. 265257 and reverse primers sequence was G759 (5-GAGGCCGCCCTGGATCTTCGAGACGA-3) and synth ID No. 265259 .The PCR mixture consisted of 1x buffer containing 1.5 mM MgCl2, 200uM dNTP,s, 25pmol primers, 1.25U of taq polymerase enzyme and 5ul of DNA in a final volume of 50ul.The PCR was performed as initial denaturation of 5 minutes at 94 0C, a set of 35 cycles was run. Each consist of 30 second at 94 0C, 30 second at 65 0C and 60 second at 72 0C followed by final extension at 72 0C.The PCR mixture was run on 1% agarose gel having ethidium bromide (6µl) and compare with marker when illuminated.

Meteorological factor

Day to day information on maximum, minimum temperature, humidity and rainfall was collected from the meteorological record office Lahore. The monthly averages for each of weather factor were collected.

Statistical analysis.

The data was analyzed by using the Univariate ANOVA, chi square test, Duncan test and Tukey's test in the different tables which were mentioned in the footnotes.

Results and Discussion

During the one-year study period from August, 2007 to July, 2008 a total of 2160 calves, cattle and buffaloes at military dairy farm, government dairy farm, gawala colonies and household dairy were examined for *Giardiasis*. Of these 50.27% (362/720) in calves, 28.05% (202/720) in cattle and 26.11% (188/720) in buffaloes were found infected with *Giardiasis*. Among calves highest prevalence was recorded in government .dairy farm (68.33%), followed by gawala colonies (55%) then military dairy farm (44.33%) and the lowest (34.44%) was recorded in household dairy. Overall highest (61.6%) season wise prevalence was recorded during autumn followed by spring (60.83%) then summer (53.4%) and the lowest(34.1%) was recorded during winter .The highest(65%) month wise prevalence was recorded during August and the lowest (30%) during December Female were found more affected (56.74%) than male (35.1%). The prevalence was significantly higher (71.52%) in younger calves than the adults (36.11%) ($P<0.05$) (Table No. 1).

Amongst the cattle, the highest prevalence (41.67%) was recorded at government dairy farm followed by gawala colonies (32.72%) then military dairy farm (22.72%) and the lowest (15%) was recorded in household dairies. The highest (35%) month wise prevalence during August and the lowest (21%) during January A female were found more susceptible (29.21%) than males (18.75%). The disease was significantly high (38.88%) in calves as compared to the adults (24.44%) (Table No. 2).

Similarly, highest prevalence (40.55 %.) in buffalo was recorded at government dairy Farm followed by gawala colonies (30%) then military Dairy Farm (21.11%) and the lowest prevalence i.e.12.77% was recorded in house hold dairies. The highest (46.66 %.) prevalence was recorded during August while the lowest (6.66%) during November and December. Females were found more susceptible than males. Where as, prevalence in younger buffaloes were significantly higher as compare to the adult (Table No. 3).

Prevalence in relation to meteorological factors indicated that there was a positive correlation of disease to minimum temperature, humidity and rainfall. Statistical analysis revealed a direct correlation between disease and rainfall.

Present study showed some epidemiological aspects of *Giardiasis* in bovines. The occurrence of *Giardiasis* at military dairy farm, gawala colonies, government dairy farm and household dairy is influenced by multifactor system that is composed of host, parasite agents, transmission process and environmental effects.

Overall prevalence of *Giardiasis* in calves was found 50.27percent. Similar finding were reported by (Deshpande and Shastri, 1981; Wade *et a.,,* 2000; Nikitin *et al*., 1991; McAllister *et al*.,.2005).

In this study, an overall prevalence of *Giardiasis* in cattle was found 28.05 percent and Similar findings were reported by (Olson *et al., 1997b ; Wad, et al., 2000)* In the present study the highest seasonal wise prevalence 31.66 percent was noted during Autumn followed by summer then Spring where as the lowest23.33 percent during winter.Similiar result were also reported by Wade *et al* ., (2000); Ralston *et a.,(* 2003) and McAllister *et al.,* (2005).

The highest overall age wise prevalence was 38.88 percent; nearly the similar results were also reported by Fabienne *et al.,* 2006. Sex wise prevalence reported in the present study was 34.62 percent in females where as 18.75 percent in males as was also reported by McAllister *et al*., 2005.In all the sites examined in the present, the highest (41,67%) prevalence was noted in government dairy farm followed by Gawala dairy colonies(32.72%), the military dairy farm (22.78%) while the lowest(15%) in house hold dairies .Results of the present study are nearly similar to Trout *et al*., 2005; Fabienne *et a.,* 2006; Wade *et al*., 2000, Ralston *et a.,* 2003 and McAllister *et al.,* 2005 Minor differences in the prevalence may be due to difference in the managemental and environmental conditions.

An overall prevalence of *Giardiasis* in buffaloes was 26.11% .Studies conducted throughout the world have indicated the wide spread of *Giardiasis* in farm animal particularly in bovines reported by Deshpande and Shastri, 1981; Nikitin *et al*., 1991; Xiao, 1994 and Fanthum, 1921 . This difference in prevalence may be due to different geographical regions and the environment. Studies in different areas showed a variation in degree of prevalence in calves. The highest prevalence was recorded in government dairy farm 68.33%, followed by gawala colonies 55% then military dairy farm 43.3% while the lowest was in household dairies 34.44%. Statistical analysis showed significant difference ($P<0.05$) among them. In British Columbia 75-83% of calves from 2-24 weeks of age on 20 different farms were positive for *Giardia* cyst (Olson *et al*., 1997a).

High prevalence was recorded in the young animals as compared to the adults. Similarly, 71.52% prevalence was recorded in 0-6 months of age group, 36.11% in 7-12 months of age. Like wise in cattle and buffaloes, the younger were more susceptible than adults. It was observed during the study that the age was an important factor in determining the prevalence of the infection and shedding of the cysts in faces. The steady drop of the infection rate from

younger animal towards old animals showed the relation ships of age and infection. The age may attributed to the development of the immunity, age related resistance and movement of the animals to a less contaminated environment after weaning. Similar results were also reported by Markovics and Pipano, 1987 and Xio and Hard, 1994. Most previous study on *Giardia* in human and bovine were based on the microscopy examination, others methods for detection of the cyst in stool specimen like enzyme immunoassay for stool antigen and *Giardia*. These assays are not enough to detect low levels of infection (Johnston *et al.,* 2003. In the present study, PCR diagnosis was higher than microscopic detection of cyst of *Giardia*. The PCR based prevalence was 31.11% in cattle as compared to direct microscopic examination 28.05%. The statistical analysis showed the significant difference was ($P<0.05$). The numbers of positive samples detected by PCR were greater than that were detected through microscopy. So the molecular technique was more sensitive in term of diagnosis (McGlade *et al.,* 2003; Mathis *et al.,* 1996). Considering the role of the meteorological data in the spread of *Giardiasis* in calves, cattle and buffaloes it was noted that the monthly distribution pattern of *Giardiasis* in bovine was related to the temperature, humidity and the rainfall. Gradual increase in average temperature 19.98-30.09°C, humidity and rain fall associated with increased incidence. The highest prevalence of *Giardiasis* in calves, cattle and buffaloes was noted during August. The mean temperature 31.48°C, humidity 71.28% and rainfall 3.2mm was noted. It was noted that with an increase of temperature, humidity and rainfall there was increase of % prevalence of *Giardiasis* in calves, cattle and buffaloes. During the months of November and January, where temperature is very low i.e. 20.41°C and 14.66°C and humidity was noted 67.09% and 65.32% respectively. The prevalence of *Giardiasis* was very low. It shows the temperature and humidity played major role in its prevalence. *Giardia* infection were more frequently diagnosed during rainy season in diarrheic patient at Bangladesh (Alam *et al.,* personal communication) where as at Saudi Arabia high prevalence in humans was reported during Autumn season. (Kasim and Elhelu, 1983).

Conclusion

The study gives the confirmation of Giardia infection in calves, cattle and buffaloes and emphasizes the need of further studies o its zoonotic strains. This study further gives the hypothesis that Giardia is the potential waterborne aetiological agent of disease in human.

Table No. 1 Overall prevalence (%) of *giardiasis* in calves at Lahore from August -07 to Jul-08

Factors		Military D. Farm		Gawala Coloney		G. Dairy Farm		Household Dairies	
		Infected / Total	Prevalence (%)	Infected /Total	Prevalence (%)	Infected /Total	Prevalence (%)	Infected /Total	Prevalence (%)
Time (Months)	Aug-07	9/15	60%♣	11/15	73%♣	12/15	80%♣	7/15	46%♣
	Sep-07	8/15	53.33%♣	10/15	66.66♣	11/15	73♣	6/15	40♣
	Oct-07	8/15	53.33%♣	11/15	73♣	13/15	86.66♣	7/15	46.6♣
	Nov-07	4/15	26.66%♣	6/15	40♣	8/15	53.55♣	2/15	13.33♣
	Dec-07	3/15	20%♣	5/15	33.33♣	7/15	46.66♣	3/15	20♣
	Jan-08	5/15	33.33%♣	6/15	40♣	7/15	46.66♣	2/15	13.33♣
	Feb-08	5/15	33.33%♣	6/15	40♣	8/15	53.33♣	5/15	33.33♣
	Mar-08	7/15	46.66%♣	10/15	66.66♣	12/15	80♣	6/15	40♣
	Apr-08	9/15	60%♣	10/15	66.66♣	12/15	80♣	7/15	46.66♣
	May-08	7/15	46.66♣	9/15	60♣	12/15	80♣	6/15	40♣
	Jun-08	6/15	40♣	7/15	46.6♣	10/15	66.66♣	5/15	33.33♣
	Jul-08	7/15	46.66♣	8/15	53.33♣	11/15	73.33♣	6/15	40♣
Season	Spring	16/30	53.33♠	21/30	66,66♠	24/30	80♠	13/30	43.33♠
	Summer	29/60	48.33♠	35/60	58.33♠	45/60	75♠	24/30	40♠
	Autumn	16/30	53.33♠	21/30	70♠	24/30	80♠	17/30	43.33♠
	Winter	17/60	28.33♠	23/60	38.33♠	30/60	50♠	12/60	34.16♠
Age	0-6 month	54/72	75	56/72	77.77	60/72	83.33	36/72	50
	6-12 month	24/108	22.22	43/108	39.81	63/108	58.38	26/108	24.07
Sex	Male	16/54	29.62	23/54	42.29	25/54	46.29	12/54	22.22
	Female	61/126	49.20	77/126	60.31	98/126	77.77	50/126	39.68

Total	78/180	43.33%	99/180	55%	123/180	68.33%	62/180	34.44%

Statistical analysis Tukey's test; Significance (P< 0.05) ♣ and Non significant (P>0.05)♠ and for season applied the *chi* square test

Table No. 2 Month Wise Prevalence of Giardiasis in Cattle at Lahore From August 2007 to July 2008

S.No.	Month	Parameter	Military form	Gawala Colonies	G. Dairy Form	Household Dairies	Total
		Total Sample Exam.	15	15	15	15	60
1	**Aug. 07**	No. Infected	4	7	8	2	21
		%age	**26.67**	**46.67**	**53.34**	**13.34**	**35%**
		Total Sample Exam.	15	15	15	15	60
2	**Sept. 07**	No. Infected	4	6	7	3	20
		%age	**26.67**	**40**	**46.67**	**20**	**33.34%**
		Total Sample Exam.	15	15	15	15	60
3	**Oct. 07**	No. Infected	4	5	6	3	18
		%age	**26.67**	**33.34**	**40**	**20**	**30%**
		Total Sample Exam.	15	15	15	15	60
4	**Nov. 07**	No. Infected	3	4	6	2	15
		%age	**20**	**26.67**	**40**	**13.34**	**25%**
		Total Sample Exam.	15	15	15	15	60
5	**Dec.07**	No. Infected	3	3	5	3	14
		%age	**20**	**20**	**33.34**	**20**	**23.34%**
		Total Sample Exam.	15	15	15	15	60
6	**Jan. 08**	No. Infected	2	4	6	1	13
		%age	**13.34**	**26.67**	**40**	**6.67**	**21.67%**
		Total Sample Exam.	15	15	15	15	60
7	**Feb. 08**	No. Infected	3	4	5	2	14
		%age	**20**	**26.67**	**33.34**	**13.34**	**23.34%**
		Total Sample Exam.	15	15	15	15	60
8	**Mar. 08**	No. Infected	4	5	6	2	17
		%age	**26.67**	**33.34**	**40**	**13.34**	**28.34%**
		Total Sample Exam.	15	15	15	15	60
9	**Apr.08**	No. Infected	4	5	6	3	18
		%age	**26.67**	**33.34**	**40**	**20**	**30%**
		Total Sample Exam.	15	15	15	15	60
10	**May.08**	No. Infected	3	6	7	1	17
		%age	**20**	**40**	**46.67**	**6.67**	**28.34%**
		Total Sample Exam.	15	15	15	15	60
11	**June. 08**	No. Infected	4	4	5	3	16
		%age	**26.67**	**26.67**	**33.34**	**20**	**26.67%**
		Total Sample Exam.	15	15	15	15	60
12	**July.08**	No. Infected	3	6	8	2	19
		%age	**20**	**40**	**53.34**	**13.34**	**31.67%**
		Total Sample Exam.	180	180	180	180	720
Overall		No. Infected	41	59	75	27	202
		%age	**22.78**	**32.78**	**41.67**	**15**	**28.05%**

Statistical analysis; Duncan test significant (P<0.05) in month wise; Non significant (P>0.05) in-group wise

Table No. 3 Prevalence (%) of *giardiasis* in buffaloes at Lahore from August 07 to July 08

Factors		Military D. Farm		Gawala Coloney		G. Dairy Farm		Household Dairies	
		Infected / Total	Prevalence (%)	Infected / Total	Prevalence (%)	Infected / Total	Prevalence (%)	Infected / Total	Prevalence (%)
Time (Months)	**Aug-07**	5/15	33.33	6/15	40.00	7/15	46.66	3/15	20.00
	Sep-07	4/15	26.66	5/15	33.33	8/15	53.33	2/15	13.33
	Oct-07	3/15	20.00	6/15	40.00	7/15	46.66	2/15	13.33
	Nov-07	2/15	13.33	5/15	33.33	6/15	40.00	1/15	6.66
	Dec-07	2/15	13.33	4/15	26.66	5/15	33.33	1/15	6.66
	Jan-08	2/15	13.33	4/15	26.66	5/15	33.33	2/15	13.33
	Feb-08	3/15	20.00	3/15	20.00	6/15	40.00	2/15	13.33
	Mar-08	4/15	26.66	4/15	26.66	5/15	33.33	2/15	13.33
	Apr-08	3/15	20.00	4/15	26.66	6/15	40.00	3/15	20.00
	May-08	3/15	20.00	4/15	26.66	6/15	40.00	2/15	13.33
	Jun-08	4/15	26.66	4/15	26.66	6/15	40.00	1/15	6.66
	Jul-08	3/15	20.00	5/15	33.33	6/15	40.00	2/15	13.33
Season	**Spring**	7/30	23.33	8/30	26.66	11/30	36.66	5/30	16.66
	Summer	15/60	25.00	19/60	31.66	25/60	41.66	8/30	13.33
	Autumn	7/30	23.33	11/30	36.66	15/30	50.00	4/30	13.33
	Winter	9/60	15.00	16/60	26.66	22/60	36.66	6/60	10.00
Age	**2-3 yrs**	12/50	24.00	16/50	32.00	22/50	44.00	8/50	16.00
	3-5yrs	26/130	20.00	38/130	29.23	51/130	39.23	15/130	11.30
Sex	**Male**	2/15	13.33	4/15	26.66	5/15	33.33	1/15	6.66
	Female	36/165	21.81	50/165	30.30	68/165	41.20	22/165	13.33
Overall		**38/180**	**21.11**	**54/180**	**30.00**	**73/180**	**40.55**	**23/180**	**12.77**

Statistical analysis; *Chi* square test; P >0.05 N

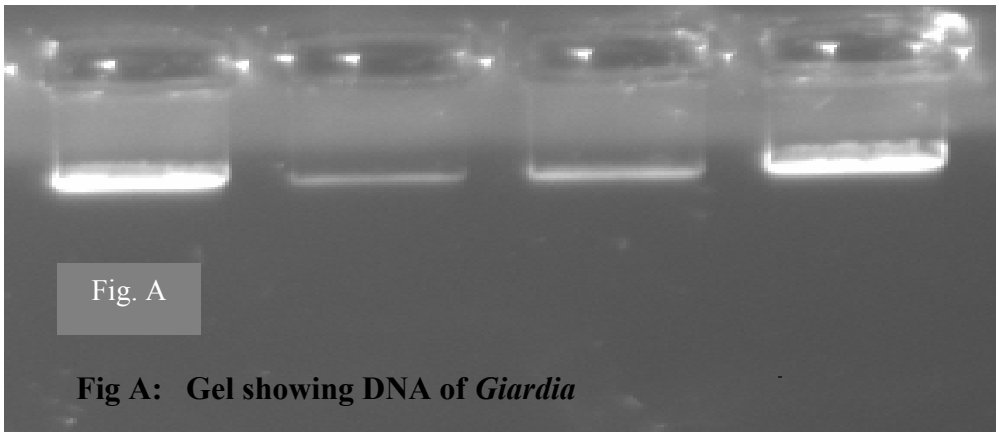
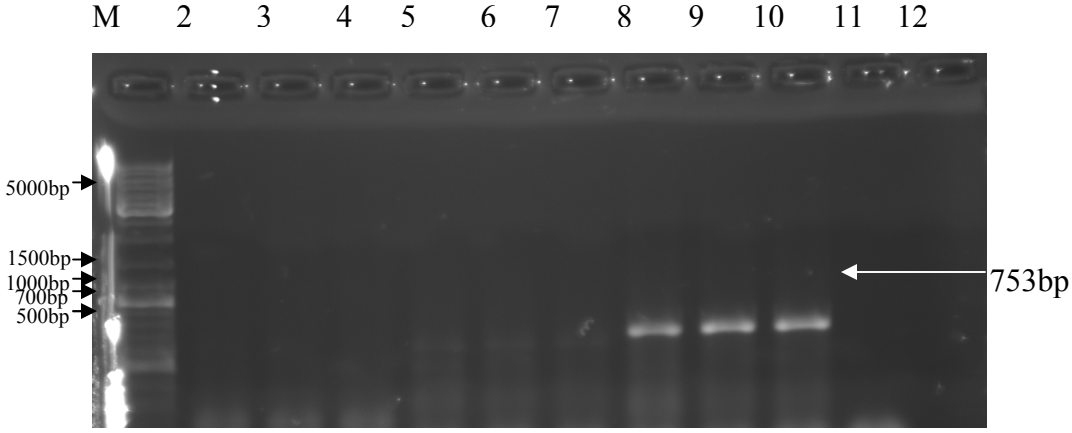


Fig A: Gel showing DNA of *Giardia*



B. *Giardia* DNA amplification by PCR Result

Lane M. 1Kb DNA Ladder Marker (Gene Ruler™ Fermentas GSY), Lane 2-7, 11 & 12: Negative samples; Lane 8-10: Positive sample (733bp product)

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