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Jake OO (2002). Pharmaceutical Interactions between *Striga hermonthica* (Del.) Benth. and fluorescent rhizosphere bacteria Of *Zea mays*, L. and *Sorghum bicolor* L. Moench for Striga suicidal germination In *Vigna unguiculata*. PhD dissertation, Tehran University, Iran.

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Full Length Research Paper

Prevalence and economic importance of liver parasites: Hydatid Cyst, *Fasciola* species and *Cysticercus tenuicolis* in sheep and goats slaughtered at Addis Ababa abattoir enterprise in Ethiopia

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This research was designed to determine the prevalence of *Cysticercus tenuicollis*, *Fasciola* and Hydatid cyst and to estimate the magnitude of the direct financial losses attributed to the condemned liver of sheep and goats slaughtered in the abattoir. Ante-mortem examination was done to determine the origin and age of animals slaughtered. Post-mortem inspection was conducted on a total of 1152 animals comprising 576 sheep and 576 goats and (288/1152) 25% livers were condemned due to parasitic induced gross lesions. From 176 (30.6%) positive sheep, 45 (7.81%) were infected by C. *tenuicollis*, 108 (18.75%) by *Fasciola* and 23 (3.99%) by Hydatid cyst. Similarly from 112 positive goats, 91 (15.8%), 13 (2.26%) and 8 (1.39%) C. *tenuicollis*, *Fasciola* and Hydatid cyst were recorded. *Fasciola* was leading cause of liver condemnation in sheep and *C. tenuicollis* in goats. Hydatid cyst was responsible for lowest condemnation rate. A statistically significant difference was observed between species and origin of small ruminants for *Fasciola*, C. *tenuicollis* and Hydatid cyst with P < 0.05. The abattoir's financial losses due to condemnation of liver by parasitic induced gross lesions accounts approximately 157,684 ETB annually.

Key words: Prevalence, liver, *Fasciola*, Hydatid cyst, *C. tenuicollis,* sheep, goats, Addis Ababa.

INTRODUCTION

Small ruminants are important domestic animals in tropical animal production system (Devendra and Mecrorey, 1990). Ethiopia has the largest livestock population in Africa, which plays an important role in the lives of its people. It owns huge number of small ruminants, about 26.1 million sheep and 21.7 million goats (CSA, 2004). The lowland part constitutes 65% the country's area where 25% sheep and close to 100% goats' population exist (Abebe, 2003).

It accounts for only 7% of the average total capital invested in livestock in the mixed crop-livestock production system, but they account on average for 40% of the cash income and 19% of the total value of subsistence food derived from all livestock production (ESGPIP, 2008).

Hence an increase in small ruminant's production could contribute to the attainment of food self-sufficiency in the country particularly in response to the protein requirement for the growing of human population as well as to enhance the export earnings. Although this sector much contributes to national economic growth, development of the sector has different constraints. These constraints

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Abbreviations: AAAE, Addis Ababa Abattoir Enterprise; **ETB,** Ethiopian Birr; **ESGPIP,** Ethiopia Sheep and Goat Production Improvement Program; **FAO,** Food and Agricultural Organization of the United Nation; **NMSA,** National Metrology Service Agency; **PACE,** Pan African Program for the Control of Epizootic diseases; **WHO,** World Health Organization.

included animal disease, poor nutrition, husbandry, and infrastructure, shortage of trained man power and lack of government policies, rampant disease and parasitism (Gryseals, 1988; PACE- Ethiopia, 2003).

Parasitic diseases in the tropics are responsible for great losses in the meat industry than any other infectious or metabolic disease (Perry et al., 2002). Like many other African countries, it is known that *Fasciola* species, Hydatid cyst and *Cysticercus teniucollis* are major parasites responsible for low productivity in Ethiopia livestock industry due to imposing poor weight gains, condemnation of organs and carcass and lower milk yield of sheep and goats (Abebe, 1995).

Fasciolosis is known to be one of the most important parasitic diseases in Ethiopia that lowers productivity in ruminants. It is caused by the genus *Fasciola*, which migrate through the hepatic parenchyma, establish and develop to the adult stage in the bile ducts. The parasite lives parts of its life in intermediate host mainly snails of the genus *Lymnaea.* Which is found in and around wet areas, such as water holes, farm animals are likely to pick up the parasite if they drink from these sources (Okewole et al., 2000)? Fasciolosis causes significant morbidity and mortality (Okewole et al., 2000; WHO, 1995).

Fasciolosis occurs worldwide in acute, sub- acute and chronic forms. Large number of young flukes causes acute swelling and congestion of the liver producing an acute paranchymatous hepatitis in which the serous capsule of the liver may be sprinkled with hemorrhages and covered with fiber. In chronic Fasciolosis of sheep, the liver becomes irregularly lobulated and distorted, but the bile ducts through thickened dilated, distended, and of bluish color (Gracey et al., 1999).

Financial losses due to ovine fasciolosis alone was estimated at 48.8 millon Ethiopian birr per year which 6.5, 48.8 and 4.7% were due to mortality, productivity and liver condemnation respectively (Negategize et al., 1993).

On the other hand, fasciolosis is an emerging zoonotic infection of humans associated primarily with the eating of water cress contaminated with metacercaria (Margrad, 1975; Rojo-Vazquez et al., 2012). Hydatidosis is a term used to describe infection of different animals species and humans with larval or metacestodes stage of *Echinococcus* species (Grant and McManus, 2003; Parija, 2004). Ungulates, including sheep, cattle, goats, pigs and horses are intermediate hosts in which Hydatid cysts occur. Adult of the genus *Echinococcus* are found in the small intestines of dogs and other carnivores (Kassai, 1999). Four species are currently recognized within the genus *Echinococcus*; *E. granulosis, E. multiloculraries, E. oligarthus and E. vogeli* (Thompson, 1986). The parasites are perpetuated in life-cycles with carnivores as definitve hosts, which harbour the adult egg-producing stage in the intestine, and intermediate host animals, in which the infective metacestode stage develops after infection with eggs (WHO, 2004).

Hydatid cyst in livestock leads to considerable economic losses due to condemnation of edible offal's primarily liver and lung (Arene, 1983). This condemnation of edible offal's primarily due to development of Hydatid in these organs (Fischer and say, 1989). This organism in liver and lung may degenerate to form cheesy mass encapsulated in multilocular may resemble tuberculosis, but the laminated cuticular membrane is still present even after the cyst has degenerate and can be readily picked up with a pair of forceps (Gracey et al., 1999).

The loss due to condemnation of organs by Hydatid cyst, particularly liver and lung in some countries is very considerable (*Gracey* et al., 1999). These losses are of especial significance in countries of low economic output, where sheep and goat production is of particular importance (Torgerson, 2002).

Though Hydatidosis, constitutes a public health problem worldwide, yet causes a particularly heavy burden in developing countries (Eckert, 1986). The distribution *E. granulosus* is higher in rural communities of developing countries where there is close contact between definitive host, the dog, and various domestic animals, acting as intermediate hosts (Eckert and Deplazes, 2004).

Cysticercus tenuicolis is a larvae of *Taenia hydatigena* which is the most important parasite of sheep and goats is found in a large number of hosts throughout the world. The intermediate host becomes infected by ingesting of proglottids or the egg passed in the feaces of the dog in pasture or feeding areas (Soulsby, 1986; Kaufmann, 1996). Larvae migrating through the liver cause hemorrhagic tracts commonly called hepatitis cysticercosa. Massive infestation can kill animals within 19 days (Reinecke, 1983).

C. tenuicollis fibrous scars resulting from the migration of the larvae lead to condemnation of the viscera and disposal of other offals to which the mature bladder worms attach. There is no human health hazard , but the liver lesions are unsightly and affect the texture of the tissue, making it unsuitable for human consumption and the economic losses associated with condemnation of affected organs are significantly high (Hall, 1986).

Thus this study was conducted to determine the prevalence of Hydatid cyst, *Fasciola* species and *C. tenuicolis* in liver of small ruminants slaughtered at Addis Ababa Abattoir; and to estimate the magnitude of the direct economic losses caused by these parasites as consequence of liver condemnation

MATERIALS AND METHODS

Study area

The study was conducted at Addis Ababa Abattoirs Enterprise (AAAE). Addis Ababa is located at 9.03° North latitude and 38.8° East longitudes with an average altitude of 2400 m above sea level. Addis Ababa covers about 54,000 ha of land with an average population of more than 3 million. It has an average temperature during winter 6°C minimum and 23 °C maximum and during summer 10°C minimum and 24°C maximum with an annual temperature of 15.9°C. It also receives an annual rain fall of 1089

mm or 91 mm per month with 60.1% annual relative humidity which ranges from 49% in February to 82% in July (NMSA, 2007).

Addis Ababa and its peri-urban areas have more than 60,000 bovine, 20,000 ovine, 7000 equine, 5,000 caprine and 330,000 avian species. The main purposes of the Abattoir are processing of one or several classes of livestock in to fresh meat for human consumption, hygienic processing and storage of meat and edible by products, ensuring close control over environmental conditions at all stages of processing and prevent the transmission of zoonotic meat borne diseases through meat inspection.

Sample selection

The study was carried out on a total of 1152 apparently healthy small ruminants (576 sheep and 576 goats) slaughtered at Addis Ababa Abattoirs (AAA). The slaughtered animals were males and females and originated from different parts of the country which include Arsi, Debre Birhan, Bale, Afar, Shoa, Ogaden, Wollo, Omo, Borena parts of Ethiopia

Study design

A cross sectional type of study was conducted December 2011 to April 2012 to determine the prevalence of fasciolosis, Hydatid cyst and *C. tenuicolis* induced lesion in liver of small ruminants slaughtered at AAAE.

Sampling and sample size determination

By using systematic random sampling methods and 95% confidence interval with required 5% precision, the sample size of both species of animals were determined by the formula of Thrusfield (1995).

$$n=\frac{1.96^2 * Pexp(1-P_{exp})}{d^2}$$

$$n=\frac{1.96^2 * 0.5(1-0.5)}{(0.05)^2} = 384*3=1152$$

Accordingly, the minimum sample size was 384 but in order to increase precision, it was multiplied by three and 1152 liver samples were taken for study.

Study methodology

Ante-mortem Inspection

Pre-slaughter examinations of small ruminants were conducted in the lairage in order to determine the species, age and origin of animals. The age grouping was performed based on arbitrary classification according to Steele (1996) and Gatenby (1991). The age grouping was based on dentition. Those which have not erupted permanent incisor teeth, were classified as young, while those with one pair or more permanent incisor teeth were classified as adults (Gatentby, 1991; Steele, 1996).

Postmortem examination

During post-mortem livers were thoroughly inspected by visualization, palpation and making systemic incisions where necessary for the presence of cysts, parasites and other abnormalities. Pathological lesions were differentiated and judged according to FAO (2000) meat inspection manual for developing countries guidelines and the results were recorded

Assessment of financial loss

The estimation of financial loss is based on the annual slaughter capacity of the abattoirs considering market demand, average market prices in local market and the rejection rates of liver. The annual slaughter rate of AAAE is 76,295 sheep and 22,673 goats with a total of 98,968. The economic loss due to liver condemnation was estimated by the formula set by (Ogunrinade and Ogunrinade, 1980) as follows:

$$EL = \Sigma Srx*Coy*Roz$$

Where

EL = Annual economic loss estimated due to liver condemnation from local market.
Srx = Annual sheep/ goats slaughter rate of the abattoir
Coy = Average cost of each sheep or goats liver.
Roz = Condemnation rates of sheep/goats liver.

Data management and statistical analysis

The data collected from the study area were recorded in the format developed for this purpose and later on entered in to Microsoft excel 2007 program and analyzed using STATA 7.0 version. Liver condemnation rates defined as proportion of condemned liver to the total number of liver examined. The data obtained during the study was subjected to chi square statistical analysis to see the association between rejection rates of liver, origin, age groups, sex and species of animals and differences were regarded statistically significant if the p value < 0.05.

RESULTS

Prevalence study

A total of 576 sheep and 576 goats were examined at AAA for the presence of Hydatid cyst, *Fasciola* spp and C. *tenuicollis.* Of these animals, the livers of 176 (30.6%) of sheep and 112 (19.4%) of goats were rejected due to parasitic causes (Table 1).

The prevalence of Hydatid cyst, *Fasciola* and *C. tenuicollis* based on species of animals indicated that the prevalence of *Fasciola* 108 (18.75%) and *C.tenuicollis* 45 (7.81%) was higher in sheep followed by Hydatid cyst 23 (3.99%). In goats, the prevalence of *C. tenuicollis* 91 (15.8%) and *Fasciola* 13 (2.26) was higher followed by Hydatid cyst 8 (1.39%). For all of the parasites identified the results indicate significant difference with $p < 0.05$. So there is high positive association between liver parasites and species (Table 2). Analysis of the result on the basis of age indicated that the prevalence of *C. tenuicollis* was higher in adult goats 78 (18.62%) than the young ones 13 (8.28%), similarly the prevalence was found to be high in adult sheep 40 (8.75%) compared to the young 5 (4.20%)

Table 1. Overall prevalence of major liver parasites in sheep and goats slaughtered at AAAE.

Species	Total examined	Total positive	Prevalence (%)
Ovine	576	176	30.6
Caprine	576	112	19.4
Total	1152	288	25

Table 2. Prevalence of major Hydatid cyst.*Fasciola* spp and *tenuicollis* based on species.

Major liver parasites	Species				
	Ovine (n=576) (%)	Caprine (n=576) (%)	Total (%)	χ^2	P-value
Hydatid cyst	23 (3.99)	8 (1.39)	31(2.69)	7.4588	0.006
Fasciola	108 (18.75)	13 (2.26)	121 (10.5)	83.4304	0.00
C.tenuicollis	45 (7.81)	91 (15.8)	136 (11.8)	17.6415	0.00
Total	176(30.6)	112 (19.4)	288 (25)		

Table 3. The prevalence of Hydatid cyst, *Fasciola* spp and *C. tenuicollis* based on age.

Species	Number of animals examined	Prevalence (%)			Total (%)
		Hydatid cyst	*Fasciola* spp	*C. tenuicollis*	
Ovine	Young (119)	3 (2.52)	14 (11.76)	5 (4.20)	22 (18.5)
	Adult (457)	20 (4.38)	94 (20.57)	40 (8.75)	154(33.7)
	χ^2	0.8478	2.7152	4.8039	-
	P-value	0.357	0.099	0.028	-
Caprine	Young (157)	1 (0.64)	0 (0)	13 (8.28)	14 (8.91)
	Adult (419)	7 (1.67)	13 (3.10)	78 (18.62)	98 (23.4)
	χ^2	0.8910	4.9836	9.1710	-
	P-value	0.345	0.026	0.002	-
Total		31 (2.69)	121 (10.5)	136 (11.8)	288 (25)

with a statistically significant difference (P < 0.05). The prevalence of *Fasciola* was higher in adult 13 (3.10%) than in young 0 (0%) among goats which is statistically significant (P < 0.05), but among sheep prevalence was higher in adult 94 (20.57%) sheep than in young sheep 14 (11.76%), which has not statistical significant difference (P > 0.05). Hydatid cyst is not statistically significant in both age groups of sheep and goats (Table 3).

The prevalence of Fasciola 101 (20.7%) and *C. tenuicollis* 39 (7.99%) was higher in sheep coming from highland areas followed by Hydatid cyst 22 (4.51%) than sheep coming from lowland areas 7(7.95%), 6 (6.82%) and 1 (1.14%) respectively. In goats coming from highland areas highest infection rate was due to *C. tenuicollis* 36 (20.11%), followed by Fasciola 10 (5.59%) and while Hydatid cyst were found to be the least frequently recorded. There was a statistical significant difference (P < 0.05) in the prevalence rates of Fasciola and Hydatid cyst and origin of animals. However, no statistical significant difference (P > 0.05) in the prevalence rates of *C. tenuicollis* and origin of animals (Table 4). Hydatid cyst, Fasciola spp and C tenuicollis are not statistically significant in both sex groups of sheep and goats (Table 5).

Estimation of financial losses

By applying the formula stated previously the annual financial loss associated with *Fasciola* spp, *Hydatid* cyst and *C. tenuicollis* are calculated as follows:

1) Annual slaughter rate of AAAE is 76,295 sheep and 22,673 goats.
2) Average rejection rate of sheep liver is 24.1%
3) Average rejection rate of goat's liver is 5.73%.
4) Average cost of sheep and goat liver is 8 birr.

Annual slaughtered sheep at AAAE was 76,295. On this study, from 576 samples 139 livers were totally condemned due to *Fasciola* spp, Hydatid cyst and *C. tenuicollis* and from 76,295 annually slaughtered animals average

Table 4. The prevalence of Hydatid cyst, *Fasciola* spp and *C. tenuicollis* based on origin.

Species	Number of animals examined	Prevalence (%)			Total (%)
		Hydatid cyst	*Fasciola* spp	*C. tenuicollis*	
Sheep	Highland (488)	22 (4.51)	101(20.7)	39 (7.99)	162 (33.2)
	Lowland (88)	1 (1.14)	7 (7.95)	6 (6.82)	14 (15.9)
	χ^2	2.2111	7.9459	0.1426	-
	p-value	0.137	0.005	0.706	-
Goat	Highland (179)	7 (3.91)	10 (5.59)	36 (20.11)	53 (29.6)
	Lowland (397)	1 (0.25)	3 (0.76)	55 (13.85)	59 (14.9)
	χ^2	12.0583	13.0519	3.6319	-
	P-value	0.001	0.000	0.057	-
Total		31 (2.69)	121 (10.5)	136 (11.8)	288 (25)

Table 5. The prevalence of Hydatid cyst, *Fasciola* spp and *C. tenuicollis* based on sex.

Species	Number of animals examined	Prevalence (%)			Total (%)
		Hydatid cyst	*Fasciola* spp	*C. tenuicollis*	
Sheep	Female (361)	18 (4.99)	62 (17.17)	29 (8.03)	109 (30.2)
	Male (215)	5 (2.33)	46 (21.40)	16 (7.44)	67 (32.2)
	χ^2	2.4881	1.5758	0.0654	
	P-value	0.115	0.209	0.798	
Goat	Female (253)	3 (1.19)	7 (2.77)	40 (15.81)	50 (19.8)
	Male (323)	5 (1.55)	6 (1.86)	51 (15.79)	62 (19.2)
	χ^2	0.1359	0.5316	0.000	
	P-value	0.712	0.466	0.995	
Total		31 (2.69)	121 (10.5)	136 (11.8)	288 (25)

rejection rate was 24.1%. One liver on local market costs 8 Birr. So, the financial losses of condemned liver due to *Fasciola*, Hydatid cyst and *C. tenuicollis* from sheep were estimated to be 147,292 Birr.

On the other hand, annual slaughtered goats at AAAE were 22,673. In the current study, 33 livers were totally condemned from a sample size of 576. From 22,673 annually slaughtered animals average rejection rate was 5.73%. One liver of sheep on local market costs 8 Birr. So, the financial losses of condemned liver due to *Fasciola,* Hydatid cyst and *C.tenuicollis* from goats were estimated to be 10,392 Birr.

DISCUSSION

An important function of meat inspection is to assist in monitoring the diseases by providing feedback information to the veterinary service to control or eradicate diseases, to produce wholesome products and to protect the public from zoonotic hazards (Gracey et al., 1999). In the present study, the prevalence of Hydatid cyst, Fasciola species and C.tenuicolis in liver of small ruminants slaughtered at Addis Ababa Abattoir was investigated and the magnitude of the direct economic losses caused by these parasites as consequence of liver condemnation was estimated. The prevalence of *C. tenuicollis* within species was higher in the liver of goats 15.8% than sheep 7.81%. This difference was found to be statistically significant. Previous studies have indicated that goats were more infected with *C. tenuicollis* than sheep (Sisay et al., 2007). According to Torgerson et al. (1998), high infestation of C. *tenuicollis*, results in the development of protective immunity early in life and this immunity regulate the parasite population, where as goat develops the immunity more slowly. This considerable degree of immunity against *C. tenuicollis* infection in sheep may be the reason for low prevalence of the parasite in comparison to goats.

The prevalence of *C. tenuicollis* in sheep and goats was relatively lower when compared to the finding of Sisay et al. (2007) in different abattoir. The prevalence was also lower than the reports from other countries. For instance, in Egypt, a prevalence of 34.5% of C. *tenuicollis*

in sheep (Abu-Elwafa et al., 2009); in Nigeria, a prevalence of 21.4% in sheep and 34.2 and 33.3% in goats (Dada and Bellino, 1978; Nwosu et al., 1996) were reported. In Iran a prevalence of 34.2% in goats, 21.4% in sheep (Solaymani et al., 2003) was also reported. The relatively lower prevalence in this study could be due to the variation in temperature, environmental condition, the degree of pasture contamination because of uncontrolled dog movement and the way of raising and grazing of these animals that may contribute to the transmission cycle between ruminants, dogs and other wild canines.

The statistical significant variation of *C. tenuicollis* prevalence between young and adult of both sheep and goats may be because that the adult animals (sheep and goats) lived longer and picked large numbers of eggs during grazing as compared to the young which only live for a short period of time.

The prevalence of *C. tenuicollis* based on animal origin was relatively higher for highland originated sheep than lowland originated sheep. Similarly, the prevalence of *C. tenuicollis* was higher for highland originated goats than lowland originated goats. The relative prevalence difference between the two areas may be due to high temperature and low humidity in the lowland area which is adverse conditions for the survival of the eggs of *T. hydatigena* and also the presence of uncontrolled movement and high dog population in highland area which is related to high human population.

The overall prevalence of liver flukes in small ruminants encountered in this study was 10.5%. When compared to previous studies (Hossain et al., 2011; and Rahmeto, 2010). This may be due to the absence of conducive ecological factors for intermediate host, snails over much of the areas where the study animals originate.

The prevalence of *Fasciola* in sheep was higher than in goats which were 18.75 and 2.26% respectively, which is statistically significant ($P < 0.05$). Previous studies, also reported similar results (Lotfi, 1995; Ezana, 2008). The difference in feeding or grazing behavior of the two species could be the responsible factor for the higher prevalence of fasciolosis in sheep than goats. Goats are browsers and do not usually graze marshy areas where there is a high chance of picking the metacercaria along with the grass.

The statistically significant variation in the prevalence of fasciolosis in sheep and goats from highland than lowland areas may be due to the existence of relatively many marshy and water logged areas as well as the presence of favourable climatic condition which metacercaria favor survival of the snail intermediate host and the metacercaria in highland. The study also showed higher prevalence of fasciolosis in adult goats (3.10%) but not found in young goats (0%) and among sheep 20.57 and 11.76%, respectively. This result is statistically significant in goats ($P < 0.05$) but not in sheep.

Statistical analysis of infection rates on the basis of sex indicated that sex has no impact on infection rate that is both male and female animals are equally susceptible to the infection. Similar results that support the present finding were reported by Mulualem (1998) and Rahmto (1992). The prevalence of Hydatidosis was statistically significant ($P < 0.05$) between sheep and goats. This difference could be due to the feeding behaviour of goats as they usually prefer browsing than grazing which may reduce the chance of acquiring the *E. granulosus* infective egg from the ground.

Age-wise prevalence of Hydatidosis showed higher prevalence in adult animals. This is in agreement with previous study finding (Helina, 2012). The study also showed higher prevalence of Hydatid cyst in highland area sheep and goat than lowland area sheep and goat. This result is statistically significant in goats ($P < 0.05$) but not in sheep.

The financial loss incurred during this study as the result of condemnation of sheep and goats livers were estimated about 157,684 ETB. According to this result financial loss associated with liver condemnation due to Hydatidosis was 26,874.6 Birr which is higher than the finding of Helina, (2012), who reported 9790.01 ETB, financial loss in the same abattoir. In this study the estimated financial loss due to Fasciolosis in small ruminants slaughtered in AAAE is very high. This finding is in agreement with previous study of Nigategize et al (1993).

CONCLUSION

This study indicated liver parasites (*Fasciola* sp., *C. tenuicollis, Echinococcus granulosus*) as cause of high liver condemnation rates in slaughtered sheep and goat. The prevalence of *Fasciola* spp, Hydatid cyst and *C. tenuicollis* varies according to age, sex, species and origin of the animals. Prevalence was significantly higher in highland than lowland animals and in adults than young animals but there was no association in prevalence of liver parasites with relation to the sex of animals. The high prevalence of parasitic diseases in liver results extensive financial loss about 157,684 ETB per annum due to the condemnation of affected livers.

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Full Length Research Paper

Hepatoprotective effect of *Phyllanthus niruri* aqueous extract in acetaminophen sub-acutec exposure rabbits

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The hepatoprotective effect of the aqueous extract of *Phyllanthus niruri* was evaluated in an acetaminophen-induced hepatotoxicity study using 24 male rabbits of the New Zealand White breed. The rabbits were randomly divided into six groups of 4 rabbits each. Group I served as a negative control group and was given only distilled water. Group II received only acetaminophen at 300 mg/kg. Groups III, IV and V were all given 300 mg/kg of acetaminophen followed by varying concentrations of the extract of *P. niruri* at 100, 50 and 25 mg/kg body weight, respectively. Group VI was the positive control for *P. niruri* and was given 100 mg/kg of the extract. The treatment was given every other day for 28 days. The most significant healing or hepatoprotective effect of the extract of *P. niruri* was seen in the group administered the extract at 25 mg/kg which showed no significant change in the liver, both grossly and histologically. In most groups, the liver enzyme assay and serum albumins and globulins levels increased slightly, except the group administered 25 mg/kg extract of *P. niruri*. This study showed that *P. niruri* has hepatoprotective properties.

Key words: Acetaminophen, hepatotoxicity, *Phyllanthus niruri*, hepatoprotective, rabbits.

INTRODUCTION

Acetaminophen, also known as paracetamol, is a widely used over the counter analgesic and antipyretic agent (Nelson et al., 2002). It is commonly used for the relief of fever, headache and other minor aches and pains. It is a major ingredient in numerous cold and flu remedies (Roberts et al., 1991). Although, it is generally safe for human use at recommended doses, acute overdoses are often seen when acetaminophen is consumed above 1000 mg per single dose and above 4000 mg per day for adults (Ohki et al., 1979), or when taken above 2000 mg per day if drinking alcohol (Ottani et al., 2006). Acetaminophen consumed at these conditions can cause potentially fatal liver damage, and in rare cases, a normal dose can do the same damage in normal individuals. The risk is however heightened by alcohol consumption (Sies et al., 1997). Paracetamol toxicity is the foremost cause of acute liver failure in the Western world, and accounts for most drug overdoses in the United States, the United Kingdom, Australia and New Zealand (Högestätt et al., 2005).

Paracetamol is derived from coal tar, and is part of the class of drugs known as “aniline analgesics”; it is the only one of such drugs still in use today (Thummel et al., 1993). It is the active metabolite of phenacetin. It is not considered to be carcinogenic at therapeutic doses (Whiteman et al., 1996). The words acetaminophen and paracetamol both come from the chemical names for the compound: para-acetylaminophenol and N-acetyl-para-aminophenol. In some contexts, it is simply abbreviated

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as APAP, for *N*-acetyl-para-aminophenol (James et al., 2001).

Phyllanthus niruri (family Euphorbiaceae) is a plant possessing several pharmacological properties (Thippeswamy et al., 2011). It is known to contain phytochemicals with antioxidant properties such as the flavonoids like niruriflavones and phenolic compounds like the triterpenoids. Faremi et al. (2008) demonstrated the antioxidant and hepatoprotective effect of the family against ethanol-induced stress in rats. *P. niruri* also contains compounds like phyllanthin, hypophyllanthin and ellagic acids whose antioxidant functional properties may include scavenging of reactive oxygen species, inhibition of generation of free radicals and chain-breaking activity (Thippeswamy et al., 2011).

The hot water leave extract of *P. niruri* is administered orally as a popular fever remedy in the Dominican Republic (Simpson et al., 2000) and is taken orally to increase appetite in the Virgin Islands and Puerto Rico (Sakaida et al., 1995; Schnellmann et al., 1999). In Tanzania, it is administered orally for treating gonorrhea (Thomas et al., 2002). The hot water extract of the dried aerial parts is used as a diuretic, antipyretic and anti-malarial in Thailand (Weis et al., 1992). The cold water extract of the leaves and roots is taken orally for diabetes, and as a diuretic in the West Indies (Stockton and Paller, 1990). The entire plant is dried and grounded in buttermilk and administered orally to treat jaundice in Fiji. The milky juice of the plant is considered good for topical application on offensive sores, cuts and bruises; it is also mixed with castor oil and applied to the eye to treat eye diseases (Salas and Corcoran, 1997). In India, juice made from the fresh plant is taken orally for urogenital disorders (Tee et al., 1986). The fruit is used externally for tubercular ulcers, scabies and ringworm, while the hot water extract of the dried plant is administered orally for curing diabetes, malaria and asthma in Ayurvedic medicine (Halmes et al., 1996; Boess et al., 1998). According to the Ayurvedic system of medicine, preparations from the plant are used for the treatment of bronchitis, leprosy, anemia, urinary discharge, anuria, biliousness, asthma, hiccups and as a diuretic. According to the Unani system of medicine, the herb is a stomachic and good for sores and useful in chronic dysentery (Agharkar, 1991; Krishnamurty, 1993). The fresh root is believed to be an excellent remedy for jaundice. A poultice of the leaves with salt is used to treat scruffy, and when without salt, it is applied on bruises and wounds. The infusion of the root and leaves is a good tonic and diuretic when taken cold in repeated doses (Oudhia and Tiwari, 2001). In many parts of India especially in the deserts, it is commonly used for the treatment of snake bite, and a major component of many popular liver tonics. The roots mixed with *Commiphora mukul* are given to camels to cure indigestion (Singh et al., 1996). Because of the increased incidences of acetaminophenabuse leading to increasing incidences of hepatotoxicity, this study was designed to evaluate the efficacy of *P. niruri* in ameliorating the hepatotoxic effects that may arise following repeated administration of acetaminophen in rabbits.

MATERIALS AND METHODS

Plant collection and identification

Fresh *P. niruri* plants were collected from Zaria, Kaduna State, Nigeria. The plant was identified according to the description of Dalziel (1937) and was further authenticated by a voucher specimen at the herbarium of Ahmadu Bello University, Zaria, Nigeria. The collected plant samples were washed under running tap water to remove sand particles. Other foreign particles that were collected alongside were sorted out. The samples were then dried in an oven at 40°C for 6 days. The dried plant samples were ground to fine powder using a mortar and pestle. An aqueous extract of the plant was prepared according to the method described by Sofowora (1993). The standard methods of Trease and Evans (1978) were used in the analysis of the phytochemical components of the plant.

Phytochemical analysis of the plant

After the collection, identification and extraction of the plant, 0.5 g of the aqueous extract was used for the screening to determine the presence of phytochemicals like alkaloids, anthraquinones, cardiac glycosides, flavonoids, saponins, steroid/terpenes and tannins. The phytochemical screening was conducted according to the standard procedures described by Trease and Evans (1978) and Sofowora (1993). The procedures for the phytochemical screening are shown below, and results summarized and shown in Table 2

Acquisition and preparation of acetaminophen

The acetaminophen used in this study was the Paracetamol D. C. Grade 90% which was donated by a reputable pharmaceutical company based in Jos, Nigeria. 5 g of acetaminophen was reconstituted in 100 ml distilled water to make a 5% solution. The preparation was made fresh whenever treatment was to be instituted. Other drugs, chemicals and reagents used in this study were of good standard and were obtained from reliable pharmaceutical shops.

Experimental animals

Twenty-four (24) male grower rabbits belonging to the New Zealand White species were obtained from the small animal experimental unit of the National Veterinary Research Institute, Vom, Jos. The animals were stabilized for a week in the small animal experimental unit of the institute for seven days prior to the commencement of the experiment. The twenty-four rabbits were randomly selected and divided into six groups of four animals each. The animals were fed on pelletized feed produced by the animal feed production unit of the research institute (NVRI feed-mill).

Acute toxicity studies

Six rabbits weighing 1.0 to 1.75 kg were randomly selected for the acute toxicity studies to determine the mean lethal dose (LD_{50}) of *P. niruri*. The six rabbits were randomly divided into three groups o

Table 1. The dosages of acetaminophen and aqueous extract of *P. niruri* administered to the groups of animals.

Group	Acetaminophen (every other day) (mg/kg)	*P. niruri* (daily) (mg/kg)
I (Distilled water only)	-	-
II	300	-
III	300	100
IV	300	50
V	300	25
VI	-	100

Table 2. Phytochemical analysis of *P. niruri.*

Phytochemical	Occurrence
Alkaloid	-
Anthraquinone	-
Cardiac glycoside	++
Flavonoid	+
Saponin	++
Steroid / Terpene	+
Tannin	+

+ = Present; - = absent.

two rabbits each. The LD_{50} was determined using the OECD guidelines. Each group was kept in a separate cage. The first group was given a single dose of 500 mg/kg of the aqueous extract of *P. niruri* and observed for 48 h. When no mortality was observed in the first group, the second group was administered 1000 mg/kg of the same extract and observed for another 48 h. Similarly, the third group was given a single dose of 2000 mg/kg of the extract when no mortality was observed in the second group, and also monitored for 48 h. The groups of animals were continuously observed for two weeks. During the acute toxicity studies, feed and water were provided *ad libitum*.

Experimental design

The study was designed to evaluate the hepatoprotective effect of the crude aqueous extract of *P. niruri* at varying concentrations in rabbits, following a sub-acute exposure of the animals to acetaminophen (APAP). During the study, twenty-four male grower rabbits were divided into six groups of three rabbits each. Group I served as a negative control and was administered only distilled water orally. Group II was a positive control for acetaminophen and was administered only acetaminophen at a dose rate of 300 mg/kg. Groups III, IV and V were the experimental groups used to test the hepatoprotective effect of *P. niruri*. All were given 300 mg/kg acetaminophen, but were treated with 100, 50 and 25 mg/kg of the aqueous extract of *P. niruri*, respectively, after 30 min of the administration of acetaminophen. Group VI was the positive control group for the aqueous extract of *P. niruri*. This group received only the aqueous extract at 100 mg/kg (Table 1). Both the acetaminophen and the plant extract were administered by oral gavage. The treatments were done for 28 days during which the experimental animal were observed for obvious clinical signs of toxicity or side effects of administered drug or extract. At the end of the experiment, each rabbit was bled via the ear vein, and blood samples collected to analyze serum liver function enzymes. The rabbits were then euthanized and the livers were collected for gross and histopathological examinations.

Statistical analysis

The values obtained were expressed as mean ± SEM. One-way analysis of variance (ANOVA) with Tukey's multiple range comparison post-hoc tests were performed on the data using the GraphPad Prism software to compare the level of significance between the test groups and controls. Values of $P < 0.05$ were considered significant.

RESULTS

Phytochemical analysis

The phytochemical analysis carried out revealed the presence of saponins, tannins, cardiac glycosides, steroids and flavonoids in the leaves of *P. niruri* as shown in Table 2.

Acute toxicity testing

During the acute toxicity study, only the clinical signs of mild toxicity were seen. None of the animals died during the two weeks of the study. Thus, the mean lethal dose (LD50) of the aqueous extract of *P. niruri* was found to be >2000 mg/kg as shown on Table 3.

Sub-chronic toxicity studies

Clinical signs

No major clinical signs were observed in the experimental animals during the course of the study. However, inactivity following the administration of acetaminophen or *P. niruri* was observed in all groups with the exception of the negative control group which took only distilled

Table 3. Result of the acute toxicity studies conducted on the aqueous extract of *P.niruri.*

P. niruri single oral dose (mg/kg)	Clinical signs	Mortality
500	Animals became inactive following *P. niruri* extract oral administration and remained dull even when touched. They resumed normal activity after about 30 min of inactivity.	0/2
1000	Animals alternated between staying stationary and slight movements for the first 30 min after dosing. They resumed normal activity, feeding and moving around about 1 h post treatment.	0/2
2000	Animals remained inactive for the first 30 min following *P. niruri* administration. They resumed normal activity after about an hour after the extracts administration.	0/2

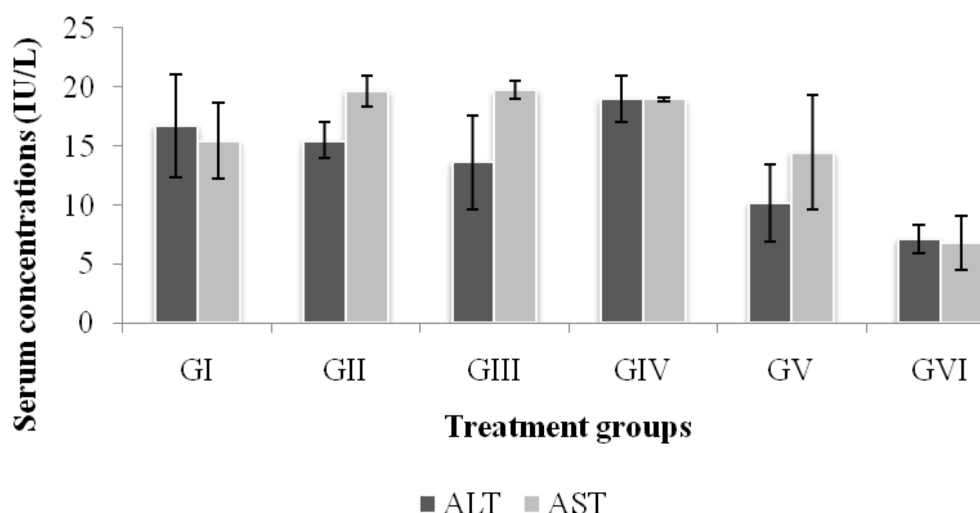

Figure 1. Mean values of serum alanine amino transferase and aspartate amino transferase.

water. Activity in animals returned to normal after about 30 min of receiving test substance.

Alanine amino transferase (ALT) and aspartate amino transferase (AST)

The mean values of the serum biomarkers of hepatotoxicity were assayed and the result showed an increase in the values of both ALT and AST within the treatment groups. These values were observed to be higher in Group II and lowest in Group VI which took only the extract of *P. niruri.* When compared within the treatment groups, the values were lower in Group V which received 25 mg/kg extract of *P. niruri.* The difference was however not significantly different when compared with the positive control group that took only *P. niruri* ($P< 0.05$) (Figure 1).

Total protein and albumin

Serum total protein concentration was highest in the acetaminophen positive control group (Group II); and lowest in Group VI. The values of serum albumin concentration were also high in Groups II and V and lowest in Group VI (Figure 2). The differences in the values of albumin were however not statistically significant when compared between all the groups ($p > 0.05$) (Figure 2).

Serum total bilirubin concentration

There was a general increase in the total bilirubin in all the treatment groups except Group I. The values of the *P. niruri* positive control group (Group VI) were also lower than those of the treatment groups. There was no

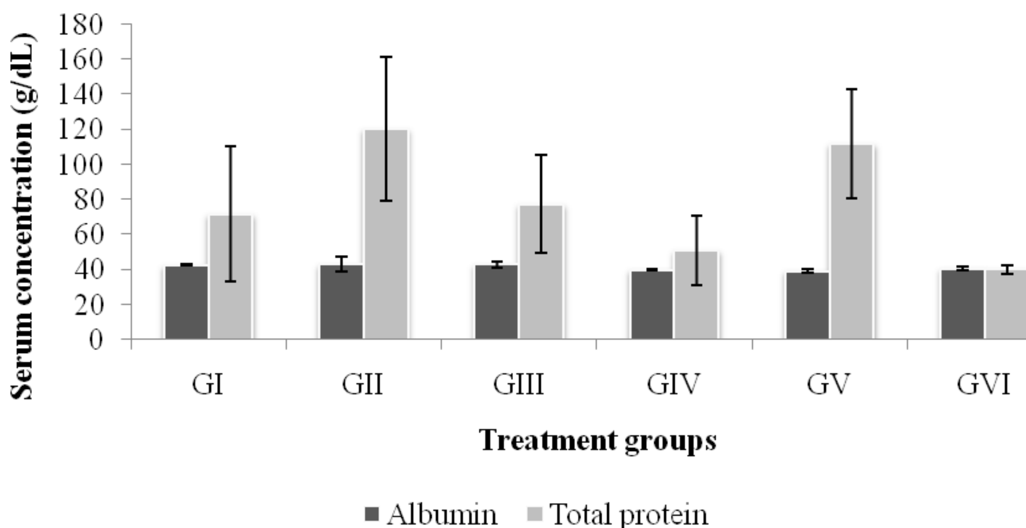

Figure 2. Mean values of serum albumin and total protein concentration.

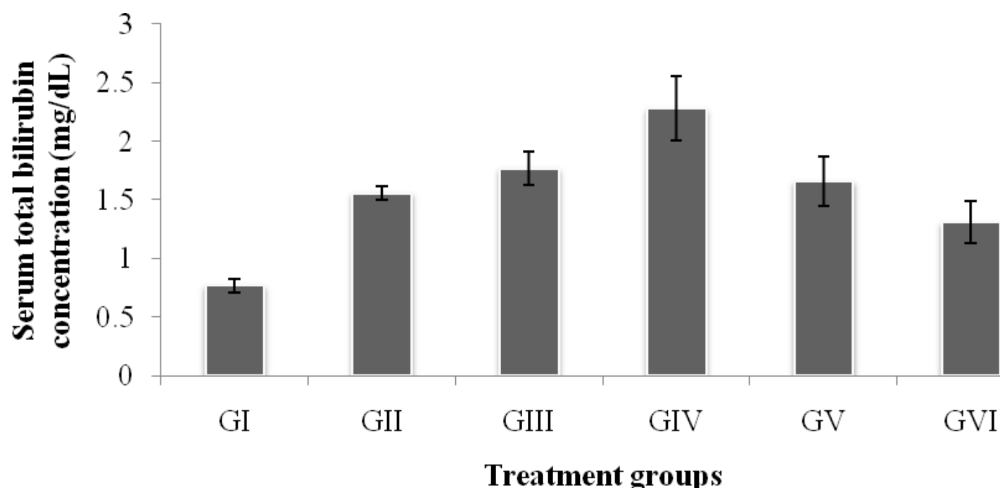

Figure 3. Mean values of serum total bilirubin concentration.

significant statistical difference between the values of all the treatment groups ($p > 0.05$) (Figure 3).

Gross pathology

When the liver samples of the treated rabbit were harvested and examined grossly, they were found to be slightly enlarged but with severe congestion. When they were incised, blood oozed out of the cut surfaces.

Histopathology

The severe congestions of the livers seen grossly appeared histologically as generalized congestion with hemorrhages. In addition, the histopathological examination also revealed the presence of centrilobular and coagulative necrosis in the livers of the animals administered acetaminophen (Figure 4c and d). The histological picture of the livers of the animals in Group V also showed congestion but with less hepatocyte damage (Figure 4b). The liver samples from the water control (Group I) and the *Phyllanthus* control (Group VI) showed normal liver architecture with no cellular damage (Figure 4a).

DISCUSSION

In this study, the mean lethal dose (LD_{50}) of the aqueous extract of *P. niruri* was found to be greater than 2000

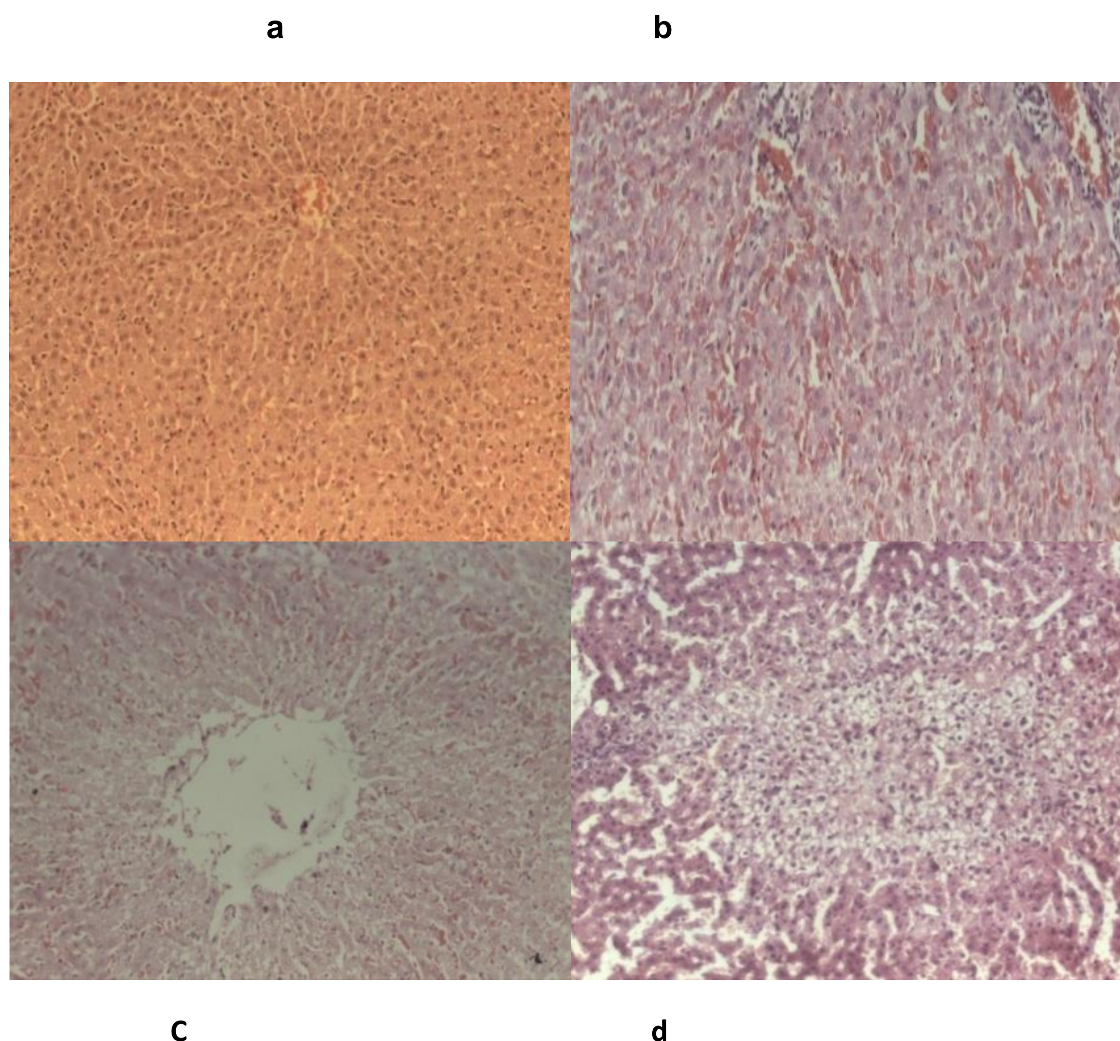

Figure 4. Histopathology of liver after H&E staining (magnification 10x). (A) Normal liver architecture; (B) Congestion with less damage to hepatocytes (Group VI); (C, D) Centrilobular and coagulative necrosis of liver, respectively (Group II).

mg/kg. The clinical signs observed in the rabbits during the study (Table 3) were thought to be physiological reactions to the newly introduced plant extract. The study showed that the aqueous extract of *P. niruri* is relatively safe in rabbits even at the concentration of 2000 mg/kg since no animal died at the end of the two weeks of the study.

The severe congestion and hepatomegaly that were seen at the gross pathological examination of the livers are indications that all the groups that were administered acetaminophen must have had liver injuries caused by the administered drug. These injuries were thought to have affected the blood vessels supplying the liver and the parenchyma, resulting in heamorrhages and congestion. This was confirmed at the histopathological examination which is used to measure and evaluate normal cellular biologic processes, pathogenic processes or biological responses to a therapeutic intervention. The histopathological examination showed the degeneration of the hepatocytes and liver parenchyma in the form of centrilobular and coagulative necrosis. These signs were found mostly in the livers of the animals in Groups II, III, and IV. These findings show that the administered acetaminophen, even at a concentration of 300 mg/kg, was capable of inducing hepatic injuries to the experimental animals. These findings were also in concordance with the findings of (Kuvandik et al., 2008, Chen et al., 2009 and Wilhelm et al., 2009). Although, the animals in Groups II, III, and IV received relatively higher doses of the extract of *P. niruri,* the hepatoprotective effect was seen more in Group V that took a lower concentration.

The increased levels of the serum liver enzymes ALT

and AST (with AST having a higher value) (Figure 1), serum albumin and total protein (Figure 2) and serum bilirubin (Figure 3) which were observed in this study also point to the fact that the administered 300 mg/kg acetaminophen produced a hepatotoxic effect in the livers of the rabbits. These enzymes are considered to be the most relevant biomarkers of liver injuries or toxicities, and thus higher levels of these enzymes within the systemic circulation points to a probable liver injury or toxicity. The findings in this study were also in agreement with previous reports made by several authors (Mazer and Perrone, 2008; Zira et al., 2009; Ghanem et al., 2009), who demonstrated that the elevations in liver markers in rats, mice and rabbits are due to liver injuries.

Generally, damage or injury to the liver impairs protein synthesis and increases serum protein concentration due to leakages from the hepatocytes (Rajesh et al., 2009). The administration of acetaminophen at (300 mg/kg) in this study produced a hepatotoxic effect that resulted in the slight leakages of these proteins from the hepatocytes into the systemic circulation. This is why these proteins were detected in the serum of the treated animals. It should also be noted that all the groups that were given acetaminophen (Groups II, II, IV and V) produced high values of AST. Although, the values of the serum albumins were low, the values of the total proteins were high in these groups (Figure 2). This shows that the liver and probably other organs were affected by the 300 mg/kg acetaminophen administered to the animals. The mean values of the serum total bilirubin concentration were found to be lowest in the negative control group, but there was no statistically significant difference when these values were compared between the treatment groups ($P > 0.05$). It should also be noted that besides Group II which received only acetaminophen, Groups III, IV and V were treated with the extract of *P. niruri* at 100, 50 and 25 mg/Kg body weight, respectively. However, the values of AST and ALT were seen to be lower in Group V which took 25 mg/kg of the extract. This result shows that the hepatoprotective or healing effect of the administered *P. niruri* was best at 25 mg/kg. The result of the liver function enzyme assay can be seen to be in concordance with the result of the gross and histopathological examinations, showing that the best hepatoprotective effect of the aqueous extract of *P. niruri* was observed in the group of rabbits administered 25 mg/kg extract (Group V). This is also in concordance with the findings of several authors (Lee et al., 2006; Frameset al., 2008; Manjrekaret al., 2008; Adeneye and Benebo, 2008).

The hepatoprotective or healing effect of the crude extract of *P. niruri* was thought to be due to the normalization of impaired membrane function activity of the liver (Gupta and Misra, 2006). This healing or normalization process might also be associated with the high tendency of the liver tissue to rejuvenate after it has been injured or damaged. This is also in concordance with (Bhattacharjee, 2006), who stated that the protein fraction of *Phyllanthus niruri* plays a protective role against acetaminophen induced hepatic disorder via its antioxidant properties.

Conclusion

The administered acetaminophen at 300 mg/kg induced a hepatotoxic effect in the rabbits which was demonstrated by the increases in some liver biomarkers. It also caused congestion, hemorrhage, coagulative, and centrilobular necrosis in the livers of the treated animals. Treatment with the aqueous extract of *P. niruri* ameliorated these effects by lowering the levels of these biomarkers especially in the group of animals treated with 25 mg/kg of the extract. This was also confirmed by the histopathological examination of livers of this groups which showed less congestion at gross examination and had less hepatocyte damage when observed histologically.

In this study, the aqueous extract of *P. niruri* proved to be capable of providing hepatoprotection against acetaminophen induced hepatotoxicity; it is therefore, possible that it can as well offer hepatoprotection against the hepatotoxicities caused by other agents.

RECOMMENDATION

It is recommended that further work should be done to quantitatively determine the active principles responsible for the hepatoprotective effect of *P. niruri*. In addition, molecular studies should be carried out to further elucidate the mechanism of action of the active compounds of this plant.

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Full Length Research Paper

Prevalence of *Listeria* species in retail quail products from Isfahan, Iran

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***Listeria* species are Gram positive, short, non sporing rods, microaerophilic. Of the six species currently recognized, *Listeria monocytogenes* is the most important as it causes a range of infections in humans and animals. This study was undertaken to determine the occurrence of *Listeria* spp. in retail quail products in Isfahan, Iran. A total of 150 samples of meat, liver, heart, kidney, and feces of quail were obtained from retail stores in Isfahan, and analyzed using standard culture methods and biochemical tests. Out of the total 150 samples, 10 (6/6%) were positive in *Listeria* spp. The occurrence of *Listeria* spp. in samples of meat, liver, heart, kidney, and feces was 10, 3, 0, 0, and 20% respectively. Only one sample was contaminated with *L. monocytogenes* (0/6%) and other samples were contaminated with *Listeria innocua* (6%).**

Key words: Quail, culture method, *Listeria* species.

INTRODUCTION

The marked increase of contamination in food industry especially meat and chicken products by pathogenic bacteria has raised a great public concern, *Listeria* species, especially *Listeria monocytogenes* has been associated with a wide variety of food sources, particularly meat and chicken (Endang et al., 2003). *Listeria* are Gram positive, facultative anaerobic, non-spore forming, rod shaped bacteria with a low C+G content. The genus consists of six species: *L. monocytogenes*, *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri*, *Listeria ivanovii*, and *Listeria grayi*. *L. monocytogenes* is the primary human pathogen; although, there have been rates of illnesses caused by *L. selegeri*, *L. ivanovii*, and *L. innocua* (Jeyaletchumi et al., 2010). *Listeria* spp. has been isolated from poultry, red meat, and meat products in many countries around the world; although, these foods have not been associated with documented outbreaks of human listeriosis. The detection of *Listeria* spp. in meat is a particular concern in terms of consumer safety, as these organisms are capable of growing on both raw and cooked meat at refrigeration temperatures (El-Malek et al., 2010).

In the past 25 years, *L. monocytogenes* has become increasingly, a dangerous bacteria as a food-associated pathogen. Because of its high case of fatality rate, listeriosis is one of the most frequent causes of death due to food borne illness.

L. monocytogenes is an intracellular pathogen affecting mainly children, pregnant women, the aged and immune-challenged individuals (Schlech, 2000; Liu, 2006). In addition, a wide variety of animals, including sheep, cattle, goats, pigs, rabbits, mice, birds, and fish are also infected. The pathogen is also responsible for *Listeria* infections that can lead to abortion, bacteraemia, sepsis, and meningoencephalitis (Sukhadeo and Trinad, 2009). The incidence of listeriosis is relatively rare and represents less than 0.1% of all food-borne illnesses, but causes infections with very high mortalities (20 to 30% deaths) (Mead et al., 1999).

The first epidemiologically confirmed food borne outbreak of listeriosis occurred in 1981 in Canada (Schlech et al., 1983) and was linked to the consumption of coleslaw. Other outbreak of human listeriosis have been

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Table 1. Main laboratory tests for the differentiation of *Listeria* spp.

Species	Hemolysis	Phosphatidylinositol phosphatase	Acid production	
			L-Rhamnose	D-Xylose
L. monocytogenes	+	+	+	-
L. ivanovii	+	+	-	+
L. seeligeri	+	-	-	+
L. innocua	-	-	V	-
L. welshimeri	-	-	V	+
L. grayi	-	-	V	-

Table 2. Distribution of *Listeria* spp. in different poultry meat product.

Sample type	Number of sample		%
	Examined	Positive	
Meat	30	3	10
Heart	30	0	0
Liver	30	1	3
feces	30	6	20
Kidney	30	0	0
Total	150	10	6/6

associated with milk (Fleming et al., 1985), soft cheese (Linnan et al., 1988; James et al., 1985), jellied pork tongue, and other foods of animal or vegetable origin (Ryser and Marth, 1999).

MATERIALS AND METHODS

Culture method for isolation and identification of *Listeria* spp

In this study, 85 samples of meat, liver, heart, kidney, and feces of quail (17 samples of everyone) were obtained from retail stores in Isfahan. All products had been properly stored or refrigerated.

Detection of *Listeria* spp. in the poultry meat products was performed according to the standard culture method (Gnanou et al., 2005). At first, poultry meat purchased was divided into 25 g portions, and the portions were placed into Stomacher bags, and were stored at 3°C or frozen at -18°C. Enrichment culturing was performed according to the ISO 11290-1 reference method (Anonymous, 1996). Samples (25 g) were aseptically added to 250 ml of *Listeria* Enrichment Broth (UVM, Difco 0223) and were pre-enriched at 30°C for 20 to 24 h, and then 0.1 ml of the pre-enriched culture was transferred to Fraser Broth (Difco 0219) at 35°C for 24 to 48 h. After selective enrichment, samples were streaked into the *Listeria* Selective Agar (PALCAM) and were incubated at 35°C for 24 to 48 h. The plates were examined for the presence of *Listeria* colonies.

From 52 poultry meat products, 12 samples which were brown-greenish and surrounded by a black halo, were transferred to Trypticase Soy Agar supplemented with 0.6% yeast extract (TSA-YE, Difco) and were incubated at 30°C for 24 to 48 h.

Biochemical tests such as the presence of catalase, hemolytic, fermentation of xylose and rhamnose, oxidase, and umbrella-shaped growth in motility in sulfide-indole-motility (SIM) medium (sulfur reduction test, indole production, motility). Gram staining was also performed on the doubtful colonies. The main tests are presented in Table 1 (Janzten et al., 2006).

RESULTS

Out of a total of 150 samples, 10 (6/6%) were positive for *Listeria* spp. *Listeria* spp. were isolated from meat, liver, heart, kidney, and feces of quail samples. Distribution of *Listeria* spp. in products considered in this study is presented in Table 2. The level of contamination of food samples by *Listeria* spp. varied. The highest rate was observed in feces samples (20%), followed by meat (10%) and liver (3%).

DISCUSSION

Meat and chicken products have been frequently contaminated with *L. monocytogenes* and may serve as means of other pathogenic organisms. The frequent occurrence of *L. monocytogenes* in meat and chicken may pose a potential risk for consumers. Human infections primarily result from eating contaminated food and may lead to serious and potentially life-threatening listeriosis (El-Malek et al., 2010). Increasing evidence suggests that substantial portions of cases of human listeriosis are attributable to the food borne transmission of *L. monocytogenes* (Low and Donachie, 1997).

Molla et al. (2004) reported that raw meat products as expected showed a high level of contamination with *Listeria*

spp. (50.6%). It is generally assumed that such products cannot be free from *Listeria*, because of the slaughter methods (evisceration) and food processing that allows greater chance for contamination. Furthermore, *Listeria* spp. are ubiquitous in the environment (Vitas et al., 2004). People handling food at different levels can also be sources of contamination.

In this study, 52 poultry meat samples were examined for the presence of *Listeria* spp. Of the total of 52 meat samples, 11 (21.15%) isolates were contaminated with *L. innocua* and only one sample was contaminated with *L. monocytogenes*. The occurrence of *Listeria* spp. in samples of meat, liver, heart, kidney, and feces were 15, 5, 0, 0, and 30%, respectively. This was comparable with results of surveys undertaken in other countries. This suggests the presence of a significant public health hazard linked to the consumption of foods contaminated with *Listeria* spp.

In this study, *L. innocua* was the predominant isolated species, and it is more frequently isolated than *Listeria* spp. When the results of this study were compared with those of other researchers, a considerably higher level of contamination of poultry raw meat from supermarkets in Spain was reported by Capita et al. (2001). *Listeria* was found in as much as 95% of examined carcasses, of which 32% of them were recognized as *L. monocytogenes* and 66% as *L. innocua.* Vitas et al. (2004) reported 36.1% positive samples of raw poultry their research carried out in Northern Spain. Kosek-Paszkowska et al. (2005) reported 63% contamination from poultry meat products. When several studies in various countries are compared, *L. monocytogenes* isolation rates seem to vary significantly. This wide variation may be explained in terms of geographic location, isolation methods, kinds of media employed and hygienic production, HACCP application, etc.

Researchers found out that *L. innocua* grows faster than the pathogenic species in enrichment broth media and may therefore overgrow than *L. monocytogenes*. Adzitey and Huda (2010) reported that *L. innocua* occupies the same ecological niche and its high incidence signifies potential contamination by *L. monocytogenes*. Higher records were reported by several investigators, such as Hassan et al. (2001) who found *Listeria* spp. in 17 (73.9%) of the 23 samples of imported frozen beef in Malaysia, Inoue et al. (2000) isolated *L. monocytogenes* in 12.2% of minced meat samples in Japan, and Buncic (1991) detected *L. monocytogenes* in 69% of minced meat samples in Yugoslavia. It is interesting to note that *L. innocua* was isolated predominantly among *Listeria* spp.

Other studies indicated that *L. innocua* was the most predominantly isolated species in a variety of meat samples; Yucel et al. (2005) reported *L. innocua* in 83.3% of the raw minced meat, 57.6% of the raw chicken meat, 63.1% of the raw beef, 9.6% of the cooked red meat, and10.7% of the cooked chicken samples. Furthermore, detection of *L. monocytogenes* in foods can be difficult as these bacteria are normally found in very low numbers in the presence of a heterogeneous micro flora. The most frequent *Listeria* isolated from food are *L. monocytogenes* and *L. innocua*. Several studies have demonstrated that *L. innocua* is found in food more frequently than *L. monocytogenes* (Walsh et al., 1998). The reasons for the higher frequency of recovery of *L. innocua* remain unclear yet. However, this may result from either a naturally higher prevalence or from preferential selection of *L. innocua* during laboratory detection procedures (Gnanou et al., 2005).

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Full Length Research Paper

Gastrointestinal helminthes of dogs and owners' perception of dogs parasitic zoonoses in Hawassa, Southern Ethiopia

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The prevalence of gastrointestinal helminthes in dogs was investigated by faecal examination from 860 dogs employing direct smear, simple flotation and sedimentation techniques. A structured questionnaire was also completed by 476 owners to assess the public awareness of zoonotic helminthes transmitted by dogs. Of the 860 dogs examined 768 (89.3%) were found to be positive for different types of helminth eggs. The following helminthes, with their respective prevalence, were diagnosed: *Strongyloides* species (60.1%), *Ancylostoma* species (52.2%), *Dipylidium* species (40.6%), *Toxocara* species (23.3%), *Echinococcus* species (5.8%) and *Trichuris* species (4.9%). The prevalence of gastrointestinal helminthes were significantly affected by age (P < 0.001), sub-city (P < 0.05) and confinement types (P < 0.001). Higher prevalence of gastrointestinal helminthes was recorded in younger dogs less than one year of age (95.6%, CI = 93.1 to 98.2). Free-roaming and semi-confined dogs were harboring significantly higher prevalence of helminthes (100%) than strictly confined dogs (62.6%, CI = 56.5 to 68.7). The present study reported that 99.2% of dog owners were not aware of the zoonotic parasite transmitted by dogs and 88.2% of them never used anthelmintics for treatment of their dogs. The high prevalence of gastrointestinal helminth parasites of dogs and lack of owners' awareness in Hawassa indicates a potential risk to human health. Thus, serious attention by the veterinarians, municipality of the town and public health service to increase awareness of their potential threat to human health is desirable.

Key words: Helminth, dogs, prevalence, zoonoses, Hawassa.

INTRODUCTION

Dogs serve as companion animals and have probably closest contact with man (Macpherson et al., 2000). The number of dogs in Ethiopian households is increasing and many families keep one or more dogs either as hunting or guard dogs. Increased numbers of dogs are seen around abattoirs, butcher shops, market places and streets (Yacob et al., 2007). Due to their closest contact with man (Robertson et al., 2000; Traub et al., 2002), gastrointestinal helminthes of dogs may be a threat to human health (Palmer et al., 2008; Dai et al., 2009) and also pose as a threat to the host: lowered resistance, retarded growth and reduced feed efficiency (Soulsby, 1982).

Free-roaming dogs are domestic dogs that are not confined to a yard or house. They have long caused major public-health problems and animal-welfare concerns in many countries (Slater, 2001). The ubiquitous problem of stray dogs in urban areas emphasizes the need to diagnose, treat and prevent zoonoses including parasitic nematodes. In Ethiopia, very few studies have been completed on (Yakob et al., 2007; Endrias et al., 2010) gastrointestinal helminthes in dogs especially in the central part of the country. Hence, there is scarcity of

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information regarding the prevalence of gastrointestinal helminthes and risk factors associated with helminth infections in the study population. Therefore, the purpose of this study was to estimate the prevalence of gastrointestinal helminthes of dogs, to identify the species of parasites and risk factors associated with helminth infections in the study population, and to assess public awareness of parasitic zoonoses transmitted by dogs in Hawassa town.

MATERIALS AND METHODS

Study area and population

The study was conducted from October 2010 to June 2011 in Hawassa, capital of the Southern Nation Nationalities People Regional State, located at an elevation of 1708 m above sea level, and between 06° 74' to 06° 8' N latitude and 38° 40' to 38° 44' E longitude. Both sexes and all age groups of dogs in Hawassa town were included in the study. For simplicity, dogs up to one year of age were grouped as young while those older than one year as adults dogs.

Sampling and sample size

To estimate the prevalence of gastrointestinal helminthes of dogs in Hawassa town 860 dogs were selected by systematic random sampling technique. The selected dogs were classified into free-roaming, semi-confined and confined based on whether they were confined or semi-confined to owner's property or homeless. The sample size for each sub-city was determined by considering 95% prevalence of gastrointestinal nematodes reported in central Ethiopia (Yacob et al., 2007). The study considered 95% level of significance (Thrusfield, 2007).

Study design

Coproscopic examination

Fecal samples were collected from 860 dogs from different sub-cities in Hawassa town and transported to the parasitology laboratory, Hawassa University School of Veterinary Medicine. The samples were examined using direct smear, simple flotation and sedimentation techniques (Hendrix, 2003; Chauhan and Agarwal, 2006). The eggs were identified based on the general characteristics described by Hendrix (2003) and Soulsby (1982).

Questionnaire for survey

The dog owners completed a semi-structured questionnaire concerning their dog's confinement types, cleaning dog's house, food source, awareness of parasitic zoonoses transmitted by dog and treatment with anthelmintics.

Data management and analysis

Data were organized, edited and analyzed using the STATA software, version 11.0 (STATA corp., College Station, TX). Descriptive statistics were used to calculate the prevalence and proportions. Chi-square test and logistic regression analysis were used to assess the association between the prevalence of dogs' gastrointestinal helminthes and the considered risk factors.

RESULTS

Prevalence of dog helminthes

Among the 860 dogs examined, 768 (89.3%) were found to be positive for gastrointestinal helminthosis. *Strongyloides* species (95% CI 56.8 to 63.4) was the most prevalent helminth infecting dogs in Hawassa, which was followed by *Ancylostoma* species (95% CI 48.9 to 55.5). Of the infested dogs, 25% (215), 37% (318), 22.3% (192), 4.5% (39) and 0.6% (5) were infested with a single, two, three, four and five species of parasites, respectively. The most commonly encountered dog parasites and their frequencies are shown in Table 1.

Risk factors for dog gastrointestinal helminthosis

There was a significant difference in the overall prevalence of gastrointestinal helminthes between the different age groups (χ^2 = 14.37, P < 0.01), among sub-cities (χ^2 = 14.37, P < 0.05) and confinement types or management (χ^2 = 257.92, P < 0.01). Gastrointestinal parasites were more frequent in young dogs, under one year of age. A significant difference was observed among the confinements types/management, and free-roaming and semi-confined dogs were more frequently infected (Table 2).

Helminth species versus risk factors

The prevalence of *Ancylostoma* species, *Strongyloides* species and *Toxocara* species significantly varied among the type of management or confinement (χ^2 = 288.31, χ^2 = 217.0 and χ^2 = 125.51, respectively, P < 0.01). The lowest prevalence of these parasites was recorded in confined dogs (95% CI = 4.4 to 16.8, 17.5 to 28.0 and 3.7 to 10.1, respectively) and the highest in free-roaming dogs (95% CI = 79.1 to 88.1, 77.8 to 87.1 and 40.9 to 53.0, respectively). Young dogs, less than one year of age, were significantly infected by higher prevalence of *Toxocara* species (χ^2 = 243.17, P < 0.01 and 95% CI for young dogs 52.3 to 65.5 and adult 6.2 to 10.6) (Tables 3 and 4).

In free-roaming dogs, significantly higher prevalence of *Echinococcus* spp. was observed than in the confined and semi-confined (χ^2 = 13.12, P < 0.05 and 95% CI for free-roaming dogs 6.6 to 14.0, semi-confined 2.1 to 6.4 and confined 1.0 to 5.5) (Table 5).

DISCUSSION

The overall prevalence of gastrointestinal helminthosis recorded was 89.3%, which is comparable to the report of Endrias et al. (2010) and Yacob et al. (2007) from central

Table 1. Prevalence of gastrointestinal helminth parasites of dogs (n = 860) in Hawassa.

Helminth parasites	Number of positive dogs	Prevalence (%)	95% CI
Nematodes			
Ancylostoma spp.	449	52.2	48.9-55.5
Strongyloides spp.	517	60.1	56.8-63.4
Toxocara spp.	200	23.3	20.4-26.1
Trichuris spp.	42	4.9	3.4-6.3
Cestodes			
Dipylidium spp.	349	40.6	37.3-43.9
Echinococcus spp.	50	5.8	4.2-7.4
Overall parasites	768	89.3	87.2-91.4

Table 2. Prevalence of dogs' gastrointestinal helminthosis and the putative risk factors.

Risk factor	Examined number	Prevalence (%)	95% CI	χ^2	P-value
Sex					
Male	688	89.4	87.1-91.7	0.04	0.85
Female	172	88.9	84.2-93.7		
Age					
Young (< 1 year)	251	95.6	93.1-98.2	14.37	<0.01**
Adult	609	86.7	84.0-89.4		
Sub-city					
Misrak	130	94.6	90.7-98.5	14.15	0.03*
Addis-Ketema	130	83.1	76.6-89.6		
Bahil-Adarash	130	84.2	77.6-90.7		
Mehal	120	92.5	87.8-97.2		
Menaharia	120	90.8	85.6-96.0		
Tabor	120	90.8	85.6-96.0		
Haik dar	120	89.2	83.6-94.8		
Confinement					
Free-roaming	262	100	-	257.92	<0.01**
Semi-confined	352	100	-		
Confined	246	62.6	56.5-68.7		

**Highly significant (P < 0.01), *Significant (P < 0.05).

Ethiopia. Eguia-Aguilar et al. (2005) and Martinez-Moreno et al. (2007) reported that more than 50% of examined dogs were infected with helminthes in Mexico City and Cordoba, respectively. Relatively lower preva-lence of dogs' gastrointestinal helminthes reported from various areas (Tylkowska et al., 2010; Balassiano et al., 2009; Palmer et al., 2008; Pullola et al., 2006; Barutzki and Schaper, 2003). The differences in health care given to dogs' and the management practice in the different geographical areas attributed to the variation in the prevalence of dogs' gastrointestinal helminthes. Treatment of dogs with anthelmintic at least once a year results in very lower prevalence (Pullola et al., 2006). Of the total examined and positive dogs, 72% were infected with multiple species, which is in a general agreement with report of Endrias et al. (2010) from Ambo, Ethiopia.

Strongyloides species was the most prevalent parasite infecting dogs in Hawassa (60.1%) followed by *Ancylostoma* species (52.2%). On the other hand, *Ancylostoma* species was the most prevalent helminth in Ambo (Endrias et al., 2010) and Debre-Zeit (Yacob et al., 2007).

With this study, the gastrointestinal helminth infection was more frequent in younger (χ^2 = 14.37, P < 0.01) and in free-roaming (χ^2 = 257.92, P < 0.01) dogs. The higher level of infection in free roaming dogs was in line with the

Table 3. Linear logistic regression analysis of confinement types and nematode infection.

Nematode species	Management type/confinements		
	Confined	**Semi-confined**	**Free-roaming**
Ancylostoma species[a]			
OR	1	9.0	35.3
95% CI	8.4-16.8	51.3-61.7	79.1-88.1
χ^2		288.31	
P-value		0.000	0.000
Strongyloides species[a]			
OR	1	7.8	15.9
95% CI	17.5-28.0	64.8-74.4	77.8-87.1
χ^2		217.0	
P-value		0.000	0.000
Toxocara species[a]			
OR	1	2.8	11.9
95% CI	3.7-10.1	13.1-21.0	40.9-53.0
χ^2		125.51	
P-value		0.000	0.000
Trichuris species[b]			
OR	1	2.1	6.7
95% CI	0.04-3.2	1.5-5.2	6.3-13.6
χ^2		20.51	
P-value		0.000	0.000

NB: a = all significantly varied, b = only confinement versus free-roaming and semi-confinement versus free-roaming significantly varied.

Table 4. Linear logistic regression analysis of age and sex, and nematode infection.

Nematode species	Age		Sex	
	Young	**Adult**	**Male**	**Female**
Ancylostoma species[a]				
OR	1.3	1	1	1.2
95% CI	51.2-65.5	46.1-54.1	41.3-56.3	49.3-56.8
χ^2	3.80			0.98
P-value	0.052			0.323
Strongyloides species[a]				
OR	1	1.1	1	1.4
95% CI	53.3-65.5	56.5-64.3	46.0-61.0	58.1-65.4
χ^2		0.08		3.89
P-value		0.772		0.048*
Toxocara species[a]				
OR	15.9	1	1.3	1
95% CI	52.3-65.5	6.2-10.6	20.6-34.0	19.1-25.4
χ^2	243.17		1.94	
P-value	0.000**		0.164	
Trichuris species[b]				
OR	1.7	1	1.3	1
95% CI	3.7-9.9	2.5-5.7	2.3-9.3	3.1-6.2
χ^2	2.57		0.38	
P-value	0.109		0.528	

NB: **Highly significant (P < 0.01), *Significant (P < 0.05).

Table 5. Linear logistic regression analysis of risk factors and cestodes infection.

Risk factor	*Dipylidium* species			*Echinococcus* species		
	OR	95% CI	P-value	OR	95% CI	P-value
Age						
<One year	1	33.4-45.5		1.3	3.7-9.9	0.447
Adult	1.1	37.1-45.0	0.662	1	3.6-7.2	
Sex						
Female	1	28.3-46.4		1	4.1-7.6	
Male	1.3	38.2-45.6	0.127	1	2.3-9.3	1
Confinements						
Confined	1	12.0-21.3		1	1.0-5.5	
Semi-confined	3.3	34.4-44.6	0.000**	1.3	2.1-6.4	0.529
Free-roaming	9.1	58.7-70.3	0.000**	3.4	6.6-14.0	0.003**[b]

NB: **Highly significant (P < 0.01), b = no significant difference only between confined and semi-confined.

observation of Komatangi (2005) and Dada et al. (1979). Free-roaming dogs had been more prone to infection due to direct and frequent contact with other dogs and their excrement and environmental contamination. Generally, no one takes care of the health of free-roaming, and no anthelmintic treatment is given in their life. Hence, once a dog is infected with certain parasite, then it remains in shedder of the eggs and contaminant of the environment for long period of time. The observed higher prevalence of gastrointestinal helminth infection in younger dogs was in a general agreement with the report of Oliveira-Sequeira et al. (2002) and Palmer et al. (2008). This higher prevalence in young dogs could be associated with their immature immune system (Bowman et al., 2003) and the transmammary transmission mode of the *Ancylostoma* species and *Toxocara* species (Urquhart et al., 1996).

Among the considered risk factors, age ($P < 0.01$), sub-city ($P < 0.05$) and confinement type significantly ($P < 0.01$) affected the overall prevalence of gastrointestinal helminthes. *Toxocara* species predominate in younger dogs ($\chi^2 = 243.17$, $P < 0.01$), which is in line with the reports from various areas (Yacob et al., 2007; Fontanarrosa et al., 2006; Pullola et al., 2006; Eguia-Aguilar et al., 2005; Oliveira-Sequeira et al., 2002). This could be associated when a bitch, once infected, usually harbor sufficient larvae to infect all her subsequent litters even if it never again encounters the infection. Trans-mammary infection of the suckling pups and once patency is established in the bitch, to contamination of the environment with eggs (Urquhart et al., 1996).

The prevalence of *Dipylidium* species significantly varied among the confinement types ($\chi^2 = 126.69$, $P < 0.01$), but the prevalence of *Echinococcus* species in free-roaming dogs varied from confined and semi-confined dogs ($\chi^2 = 13.12$, $P < 0.01$). This is mainly associated with the fact that the free-roaming dogs do not receive any type of health care and frequently infested with fleas and lice. Living as free-roaming could give chance for free movement and wandering, which is a risk of greater chance of direct contact with contaminated environments. These conditions increase the pressure infection in freely wandering dogs.

Most of the dog owners in Hawassa had awareness only about rabies public health importance, but not zoonotic helminthes transmitted by dogs (Table 6). That is why there was improper disposal outside of residences compound and open garden, of dogs faeces practiced by 94.3% of the people. Also 88.2% of them never used anthelmintics for treatment of dog helminthosis. These conditions are associated with increased contamination of the environment with helminth eggs that passed in faeces of infected dogs. From such environment, free-roaming and semi-confined dogs get the infection and hence higher prevalence of helminthosis encountered in dogs. So there is great risk of human infection, especially children playing in the open garden, by the zoonotic parasites and the exposure of human being, children, is proportional to the extent of environmental contamination (Eguia-Aguilar et al., 2005; El-Shehabi et al., 1999).

Conclusion

The prevalence of dogs' gastrointestinal parasites in Hawassa town is very high, suggesting the absence of health care given for dogs and increased number of free-roaming dogs. There was almost no owners' awareness of the dogs' parasitic zoonoses and this was manifested by the improper disposal of dogs' faeces. These have had a significant impact on the epidemiology of the gastrointestinal helminthes of dogs and a serious public health problem. All kind of dogs, confined and free-roaming, plays a role in transmission of zoonotic parasites transmitted by dogs. *Ancylostoma* species, *Strongyloides* species, *Toxocara* species, *Dipylidium*

Table 6. Summary of dogs' management and owner's perception of zoonotic diseases.

Factor	Frequency	Percentage
Housing		
Free in the compound	171	35.9
Tied or confined in 'kennel'	305	64.1
Frequency of cleaning		
Every day	21	4.4
Every week	214	44.9
Every month	11	9.2
Not at all	197	41.4
Disposal of dog's faeces		
Outside of the compound	226	47.5
In the open garden	223	46.8
Buried	19	4.0
Dumped in the toilet	8	1.7
Food source		
Household leftover	273	57.4
Raw animal product	140	29.4
Both	63	13.2
Tendency of cooking meat for dogs		
Yes	28	5.9
No	448	94.1
Public health risk awareness		
No	21	4.4
Only about rabies	451	94.7
Gastrointestinal helminthes	4	0.8
Awareness and use of anthelmintics		
No	420	88.2
Yes	56	11.8

species and *Echinococcus* species are the most relevant in terms of their zoonotic potential. But data on human infection with these parasites in the study area are lacking. Hence it requires serious attention towards this problem by the veterinarians, municipality of Hawassa town and public health service in order to reduce the level of helminthes infestation and protect the public health. Public education of the dogs' health care and other management practices should be instilled. Also, monitoring free-roaming dogs could play a key role in the controlling and reducing the prevailing problem. Further epidemiological study should be conducted to investigate the rate of seasonal infection and the level of environmental contamination.

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Full Length Research Paper

Comparison of reverse line blot and β-tubulin targeted nested polymerase chain reaction (PCR) techniques in the detection of *Theileria* and *Babesia* piroplasms in cattle in Uganda

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Three hundred (300) blood samples from apparently healthy cattle were collected in areas around Lake Mburo National Park in Western Uganda. These were used in the comparison of the sensitivity and specificity of reverse line blot (RLB) and β-tubulin targeted nested polymerase chain reaction (PCR) in the detection of tick-borne piroplasms in cattle. The sensitivity of RLB technique for the detection of *Theileria* and *Babesia* species was 71.25% (95% CI: 60.05 to 80.82%) and the specificity was 57.50% (95% CI: 40.89 to 72.960%) while the sensitivity of the beta-tubulin targeted nested PCR was 62.5% (95% CI: 50.96 to 73.08%) and the specificity was 72.5% (95% CI: 56.11 to 85.40%). The positive predictive value using RLB was 77.03% (95% CI: 65.79 to 86.01%) and the negative predictive value was 50% (95% CI: 34.90 to 65.10%) while the positive predictive value using β-tubulin targeted nested PCR was 81.97 % (95% CI: 70.02 to 90.64%) and the negative predictive value was 49.15% (95% CI: 35.89 to 62.50%). The Kappa statistic for level of agreement in detection of tick-borne piroplasms between RLB assay and β-tubulin targeted nested PCR was 0.7984 which indicated substantial agreement between the two tests. The RLB assay allowed the detection of individual species that simultaneously infected the cattle. However, it was not possible to identify the species with the β-tubulin targeted nested PCR.

Key words: *Babesia, Theileria,* β-tubulin, reverse line blot, sensitivity, specificity.

INTRODUCTION

In Africa and specifically in Uganda, tick-borne diseases (TBDs) are considered to be the major constraints to livestock productivity (Young et al., 1988; Ocaido et al., 2005). TBDs affect 80% of the world's cattle population and are widely distributed throughout the world, particularly in the tropics and subtropics. In acute cases, bovine piroplasmosis can be diagnosed by microscopic examination of Giemsa-stained thin blood smears and by clinical symptoms. Nevertheless, following acute infections, recovered animals frequently retain subclinical infections (carriers). Serological methods are employed in diagnosing subclinical infections, but false positive and false negative results are commonly observed due to cross-reaction. Therefore, a more specific and sensitive method for the diagnosis of piroplasms is required. Recently, species-specific polymerase chain reaction (PCR) and PCR-based reverse line blot (RLB) hybridization methods have been developed and used (Schnittger et al., 2004; Aktas et al., 2005).

RLB hybridization is a laborious and expensive technique that has been used for screening of tick-borne infections. Therefore, there is a need to adopt a less laborious and cheap technique that can also simultaneously detect multiple infections. All eukaryotic cells are

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structurally and functionally supported by hollow proteinacious organelles known as microtubules. Microtubules are composed of two major proteins, α and β-tubulin, and a third minor species the γ-tubulin (Little and Seeheus, 1988; Oakely and Oakely, 1989). The β-tubulin gene is one of the few apicomplexan genes that are interrupted by one or more introns (Nagel and Boothroyd, 1988). In addition, the position of the first intron is conserved in all the species investigated so far, allowing for a rational design of primers around this region. Introns associated with the β-tubulin gene show extensive variations both in length and in sequence. These features make this region of the genome a good candidate for the development of informative markers, as it has been shown previously for several protozoan parasites (Costa et al., 1997).

A fragment of the β-tubulin gene has been amplified and sequenced from *Theileria* and *Babesia* species. The presence (within the amplified gene fragment) of an intron that varies extensively both in length and in sequence, has allowed the development of an assay to differentiate the species directly on the basis of the specific size of the PCR products or by employing a simple PCR-restriction fragment length polymorphism (RFLP) protocol (Caccio et al., 2000). The ß-tubulin targeted nested PCR falls into this category of simultaneously detecting multiple infections (Caccio et al., 2000) and the detection of the reaction product can easily be done by running an agarose gel or any other method such as optical density measure, hence the necessity to compare it with RLB technique.

This study compared the capacities of ß-tubulin targeted nested PCR and RLB techniques in detection and identification of *Theileria* and *Babesia* species in healthy cattle so that one of the two can be adopted in the screening and epidemiological studies of tick-borne infections in resource poor countries.

MATERIALS AND METHODS

Blood samples

Three hundred (300) blood samples from apparently healthy adult Ankole cattle raised on free range ranging around Lake Mburo National Park (Western Uganda) were collected by jugular venipuncture into Ethylenediaminetetraacetic acid (EDTA) coated vacutainers. Purposive random sampling was used in this study. Upon collection, samples were put on ice packs and transported to the laboratory or thin smears were made and also transported to the laboratory where they were methanol fixed for 5 minutes, Giemsa stained for 30 min and then microscopically examined.

DNA extraction

Eighty (80) of microscopically positive and 40 negative samples were selected and blood aliquoted in 1.5 ml eppendorf tubes andstored at -20°C till used for DNA extraction. DNA was extracted as described by d'Oliviera et al. (1995) with some modifications. Briefly, 200 µl of thawed blood in an eppendorf tube was washed 3 to 5 times by mixing with 0.5 ml Phosphate buffered saline (PBS) (137 mM NaCl, 2.6 mM, KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), followed by centrifugation at maximum speed (13,000 rpm) for 5 min. After the final wash, the cell pellet was resuspended in 100 µl of lysis mixture (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5% Tween 20, 100 µg/ml of proteinase K). This mixture was incubated over night at 56°C, followed by 10 min of boiling to inactivate proteinase K. The mixture was then kept at -20°C until needed for PCR.

PCR amplification of 18s ribosomal RNA (rRNA)

One set of primers was used to amplify a 390 to 430 bp fragment of the 18S rRNA gene spanning the V4 region of *Theileria* and *Babesia* organisms. The forward primer used was RLB-F2 5'-GAC ACA GGG AGG TAG TGA CAA G-3' and the reverse primer was RLB-R2 5'-Biotin-CTA AGA ATT TCA CCT CTG ACA GT-3', as described by Georges et al. (2001). The primers were manufactured by Bioneer, Germany. The reaction constituents in a final volume of 25 µl were as follows: 1× PCR buffer (Invitrogen), 3.0 mM $MgCl_2$ (Invitrogen), 200 µM each dATP, dCTP, dGTP, 100 µM dTTP (ABgene) and 100 µM dUTP (Amersham), 1.25 U of Taq DNA polymerase (Invitrogen), 0.1 U of UDG (Amersham), 25 pmol of each primer, 2.5 µl of template DNA and sufficient distilled water to top up the reaction (Bekker et al., 2002). Each time, positive control DNA (*T. parva* or *B. bovis*) and negative control (reaction constituents without DNA or with *Trypanosoma* DNA) were included. The reactions were performed using the program described by Bekker et al. (2002). Thereafter, PCR products were kept at -20°C until needed for reverse line blot hybridization.

PCR amplification of beta-tubulin regions

A fragment of the β-tubulin gene was amplified using the forward primer F34 (5'-TGTGGTAACCAGAT(t/c)GG(a/t)GCCAA-3'), and the reverse primer R323 (5‟-TCnGT(a/g)TA(a/g)TGnCC(t/c)TT(a/g)GCCCA-3'). The reaction mixture and PCR amplification were as described by Caccio et al. (2000). Amplifications were performed on a Perkin-Elmer model 2400 thermal cycler. For nested PCR reactions, a forward primer F79 (5‟-GA(a/g)CA(t/c)GGnATnGA(t/c)CCnGTAA-3''), and a reverse primer R206 (5‟-AC(a/t/g)GA(a/g)TCCATGGT(a/t/g)CCnGG(t/c)T-3'') were used. The reactions were run using the same profile as described above for the primary PCR, (Caccio et al., 2000). The PCR products were run on 2% agarose, stained with ethidium bromide and visualized under UV light.

Application of species-specific oligonucleotide probes onto the biodyne C membrane

The species-specific probes were applied to the membrane as described by Gubbels et al. (1999). After activation, the membrane was sealed in a plastic bag containing 5 ml of 20 mM EDTA and stored at 4°C until required.

Reverse line blot hybridization of PCR products

Hybridization of PCR products to the species-specific probes was performed as described by Gubbels et al. (1999). Membranes were then incubated for 1 min at room temperature in 10 ml of mixed Electrogenerated chemiluminescence (ECL) detection liquids 1 and 2 (Amersham). The membrane was placed between two colorless polythene sheets, placed on the intensifying screen in an exposure

cassette with the DNA side up and exposed to an ECL-hyper film (Amersham) for 20 to 25 min in the dark room.

Data analysis

The RLB and nested beta-tubulin data sets were analyzed using the DAG-STAT software program for comparing diagnostic tests and determining the level of agreement between tests (Mckinnon, 2000).

RESULTS

Light-microscopy examination of thin blood smears

Of the 300 samples microscopically analyzed, 120 were positive (intra-erythrocytic parasites observed) and the rest (180) negative (no intra-erythrocytic parasites observed). Some samples (80 positive and 40 negative) were randomly selected and analyzed using the RLB and β-tubulin nested PCR assays.

Reverse line blot analysis

The RLB assay allowed the detection of individual species that simultaneously infected the cattle. Out of the 80 samples positive by microscopy, RLB confirmed 57 (71.25%) positive for at least one species while 23 (28.75%) were negative (Table 1). Of the 57 samples positive by RLB assay, 8 (14.04%) were infected by *T. mutans* only, 47 (82.46%) were simultaneously infected with *T. parva* and *Theileria* spp. (buffalo) but not with any other species, while 1(1.75%) sample was simultaneously infected with *T. parva*, *Theileria* spp. (buffalo) and *B. bovis.* Only 1 (1.75%) sample failed to match with any of the species represented on the probe membrane. On the other hand, out of randomly selected 40 microscopically negative samples, 17 (42.5%) were found to be positive and 23 (57.5%) maintained the negative status. Of the 17 samples that changed status to positive by RLB, 11(64.5%) had mixed infection, while 6 (35.3%) had single infection. All the samples that showed mixed infection included *T. parva* and *Theileria* spp. (buffalo) (Figure 1). Out of the 6 samples showing single infection, 5 samples were positive for *T. parva* while one was positive for *B. bovis*.

Beta-tubulin targeted nested PCR analysis

Eighty (80) samples positive by microscopy were subjected to beta-tubulin nested PCR analysis. Fifty samples (62.5%) tested positive while 30 samples (37.5%) tested negative with beta-tubulin nested PCR analysis (Table 1). The gel (Figure 2) shows some representative samples tested with the beta-tubulin nested PCR analysis. Two to three bands (300 to 400 bp) were identified in some samples, suggesting mixed infection in some samples, though it was not possible to know the specific species in question. Forty (40) samples testing negative by microscopy were subjected to beta-tubulin nested PCR analysis. Eleven samples (27.5%) were found positive and the rest negative. The beta-tubulin primers did not amplify *Trypanosoma* tubulin (Figure 2). The amplicons were obtained using primers targeting the β-tubulin DNA of *Theileria* and *Babesia* species and fractionated on a 2% agarose gel.

Comparison of β-tubulin targeted nested PCR with RLB assay

The sensitivity of RLB technique for the detection of *Theileria* and *Babesia* species was 71.25% (95% CI: 60.05 to 80.82%) and the specificity was 57.50% (95% CI: 40.89 to 72.960%) while the sensitivity of the beta-tubulin targeted nested PCR was 62.5% (95% CI: 50.96 to 73.08%) and the specificity was 72.5% (95% CI: 56.11 to 85.40%). The Positive predictive value using RLB was 77.03 % (95% CI: 65.79 to 86.01%) and the Negative predictive value was 50% (95% CI: 34.90 to 65.10%) while the Positive predictive value using beta-tubulin targeted nested PCR was 81.97 % (95% CI: 70.02 to 90.64%) and the Negative predictive value was 49.15% (95% CI: 35.89 to 62.50%). The Kappa statistic for level of agreement in detection of tick-borne piroplasms between RLB assay and beta-tubulin targeted nested PCR was 0.7984 (95% CI: 0.6925 to 0.9044).

DISCUSSION

The aim of this study was to compare Reverse line blot and beta-tubulin targeted nested polymerase chain reaction in the detection of tick-borne piroplasms of apparently healthy cattle in Western Uganda so that their role in routine diagnosis of clinical cases and in epidemiological studies can be substantiated. The RLB assay is currently used in the detection of tick-borne infections whereas the β-tubulin targeted PCR is not.

Previous studies (Gubbels et al., 1999; Oura et al., 2004; Schnittger et al., 2004) have emphasized the high sensitivity of the RLB assay. However, the RLB assay is expensive and laborious. In this study therefore, the β-tubulin targeted nested PCR was compared with the RLB assay to determine if it can be an easy but still sensitive alternative to use. The RLB permitted simultaneous detection of multiple infections in single animals as previously reported by Muhanguzi et al. (2010). A substantial proportion of the cattle showed mixed infection (84.21%) with the majority (82.46%) infected with two species only; that is, *T. parva* and *Theileria* spp(buffalo) while eight cattle (14.04%) were infected with *T. mutans* alone while one animal had mixed

Table1. Comparison of reverse line blot and the beta-tubulin targeted nested PCR with microscopy as the reference test.

TEST		Microscopy (n = 120)	
		Positive (80)	Negative (40)
RLB	Positive (74)	57	17
	Negative (46)	23	23
ß-tubulin nested PCR	Positive (61)	50	11
	Negative (59)	30	29

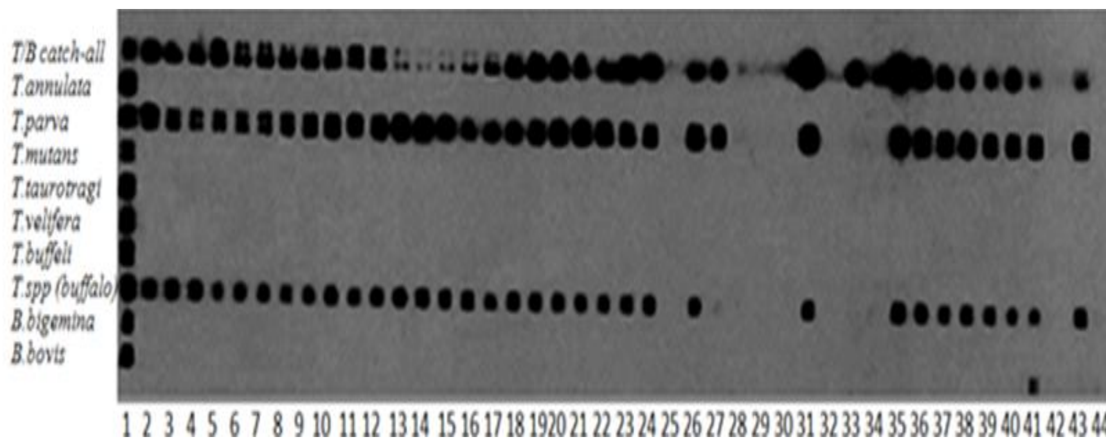

Figure 1. Reverse line blot analysis, species-specific oligonucleotide and theileria/babesia catch-all were applied to the horizontal rows of the RLB as shown. Slot 1 is the membrane positive control (Isogen, The Netherlands) and slot 27 is the positive control(*T. parva*). The remaining slots are test samples.

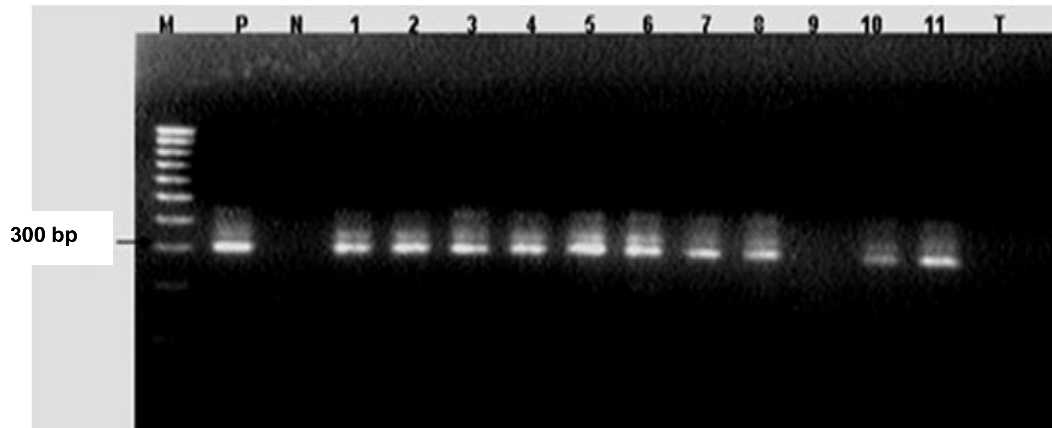

Figure 2. Agarose gel analysis of the β-tubulin PCR amplicons. Lane M; 100 bp sigma molecular weight DNA marker, lane P; positivecontrol, lane N; negative control, lanes 1 to 11,test samples and lane T; *Trypanosoma* DNA.

infection with three species that is, *T. parva*, *Theileria* spp (buffalo) and *B. bovis.* However, *Theileria* spp (buffalo) and *B. bovis* presented with weak signals. Both *T. parva* and *B. bovis* are pathogenic to cattle. The beta-tubulin nested PCR was able to determine the genus but it was not possible to determine the species. This is because the sizes of PCR amplicons of most species have not been established. The previous study by Caccio et al. (2000) used known species of *Theileria* and *Babesia* and the sizes of their PCR products were determined. The beta-tubulin primers did not amplify *Trypanosoma* tubulin DNA. This showed that the primers were specific to

Theileria and *Babesia* tubulin and probably not to any other parasite tubulin.

The beta-tubulin targeted nested PCR compared very well with the RLB assay and with very high sensitivity and specificity. Since the two tests showed substantial agreement, we strongly believe that the less laborious beta-tubulin targeted nested PCR can be used for diagnosis of clinical cases. However, for field epidemiological studies in which establishment of the species are required, the RLB would be preferable. Therefore, more research needs to be done on the beta-tubulin targeted nested PCR using known species of *Theileria* and *Babesia* such that the species-specific sizes of amplicons can be established.

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