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DEVELOPMENT OF REFERENCE TEMPLATE FOR LAMP ASSAY IN DETECTING FGCATB3 GENE IN JUVENILE FASCIOLA GIGANTICA FLUKES AMONG LARGE RUMINANTS IN AURORA, PHILIPPINES

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Abstract:

The study focused mainly on developing and validating the positive control from the cDNA of the gene expressing the cathepsin B3 enzyme secreted by the juvenile fluke of Fasciola gigantica in large ruminants only. The aim is to develop a positive control of the gene expressing cathepsin B3 gene from immature Fasciola gigantica flukes for prepatent detection among large ruminants. RT-LAMP optimization to detect Cathepsin B3 in marita of an infected liver showed optimal temperature and reaction time for amplifying FgCatB3 gene at 62C for 90 minutes, respectively, as confirmed in the dye, fluorescence test, gel electrophoresis, and RT-PCR. Field validation of the RT-LAMP protocol was done on swamp buffaloes, and cattle blood samples to detect the FgCatB3 gene showed a positivity rate of 27.5 % based on dye, fluorescence tests, and gel electrophoresis. Prepatent detection of fasciolos is in large ruminants can be possible using the optimized RT-LAMP that can amplify that gene expressing the cathepsin B3 enzyme, which is secreted exclusively by juvenile flukes. Blood samples can be examined for prepatent detection. Hence, strategic deworming can be implemented before the flukes reach the adult stage and obstruct the bile ducts leading to death. (Justify, Calibri 8)

Index Terms: RT-LAMP, RT-PCR, Fasciola gigantica, marita, flukes, FgCatB3, cDNA.

1) INTRODUCTION

Fasciola hepatica and gigantica are two liver flukes commonly reported to cause fasciolosis in ruminants (Walker et al., 2007 as cited by (al 2016) Yusuf [2016]). The infection is cosmopolitan, where Fasciola hepatica prevails in temperate areas and Fasciola gigantica occurs in tropical countries. Fasciola gigantica is the common fluke prevailing Philippines. Given warm and wet conditions, it releases the miracidia, which then invades a suitable snail intermediate host, commonly Lymnea auricularis rubiginosa or the birabid snails in the Philippines (Hulipas 2016). The newly excysted juvenile (NEJ) is the first stage in contact with the host (Campos,2016), destroying liver tissue and causing bleeding during migration. This stage causes the NEJ to release an enzyme protease known as Cathepsin B (McDougall, 2012). Cathepsin proteases enzymes play a significant role in parasitism (Jardim 2009) and even death. Fasciolosis is a disease of economic importance, with livestock impact the industry of agriculture. Human infection is now considered of public health importance and is hyperendemic in some world areas

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(Gilmar S. Erzinger, 2012). Regions with frequently flooded livestock grazing like Aurora province are prone to liver fluke infection. This is because snails that act as intermediate hosts wants small humid habitats (Junquera, 2017). This geography is one of the major factors of high mortality causing fasciolosis, especially in large ruminants in the province, due to the presence of many creeks and water banks, where we commonly see snails, particularly the Lymnaea spp., and the intermediate hosts of Fasciola. Several applications of molecular biology techniques have been developed to study genetics, diagnostics, and drug resistance in veterinary physiology (Alexio et al., 2015). Parasitic infections are detected through LAMP assay method (Adams et al., 2010; Ni et al., 2014; Martinez-Valladares and Rojo-Vazquez, 2016) as cited by Balondo, 2017. (Justify, Calibri 9.5)

Objectives

The main objective was to develop a positive control of the gene expressing cathepsin B3 gene from juvenile Fasciola gigantica flukes for prepatent detection among large ruminants of Central Aurora. The study specifically aimed to:

1. develop cDNA positive control of the gene expressing cathepsin B3 enzyme from juvenile flukes of Fasciola gigantica harvested from the infected liver of large ruminants;

2. Validate the cDNA positive control using the Reverse Transcription Loop-mediated isothermal amplification and Reverse Transcription-Polymerase Chain Reaction; and

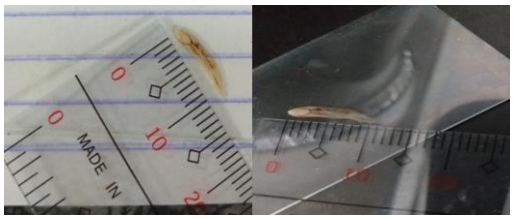
3. Conduct RT-LAMP assay field validation using the developed cDNA positive control among large ruminants in Central Aurora.

2) MATERIALS AND METHODS

Reference Template from Immature Flukes

The collected juvenile or Marita flukes which measure around 2 mm **<** 10 mm from the infected livers were extracted using a knife, scalpel, and flashlight. They were placed on a Petri dish for microscopic examination and were finely macerated for RNA extraction. Young flukes measure a few millimeters within the hepatic, while adult flukes measure 20-40mm in length and 8-13mm in width in Fasciola hepatica (Dusak, 2012) within the bile ducts see Figure 1.

Figure 1: Juvenile fluke extracted under the microscope



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RNA Extraction of Immature Flukes using Nucleozol with Revisions

Eight immature flukes were separately macerated vigorously for 15 seconds with a mini-tissue pestle in a 1.5 ul microcentrifuge tube with Nucleozol®. Incubation of tubes were done at 15 minutes at room temperature then 15 minutes centrifugation at 120,000 pm. The aqueous phase of the mixture was moved to a tube containing isopropanol and room temperature incubation for 10 minutes. The tubes centrifuged again then the contents were discarded. Around 125 ul of ethanol was put on into each tube, and centrifugation of tubes were done for three minutes around 80,000 pm. The content was discarded, and 30 ul of distilled water was added. RNA samples were stored at -80^{o}C until used. The concentration of RNA samples (ng/μl) was measured using the nano-spectrophotometer.

Blood Sample Collection and Extraction

Four municipalities of Aurora province were the sites of sample collection. These are Baler, Dipaculao, Maria, and San Luis. There were 20 cattle and 20 swamp buffaloes (carabao) enrolled in each municipality which was randomly selected based on the reported cases of fasciolosis from the Provincial Veterinary Office. The animals were distributed according to age groups: 10 cattle/swamp buffalo for those below 1-year-old and ten cattle/swamp buffalo above 1-year-old. So, for the entire study, there were 160 animals examined for Cathepsin B3. Two milliliter of blood from the ear vein was collected aseptically. Blood was placed in a vial with an anticoagulant for whole blood. All of the samples were placed in a cooler during transport. Upon reaching the laboratory, the blood samples were immediately subjected to RNA extraction.

RNA Extraction of Blood Samples

An aliquot of 200 ul of blood sample was placed in individual microcentrifuge tubes containing 500ul of Nucleozol®. It was vortexed for fifteen seconds and incubated at fifteen minutes at room temperature, and centrifuged for 15 minutes at 12000 pm. The aqueous phase was placed into a new tube containing 500ul of isopropanol. Incubation for 10 minutes was done at room temperature and centrifuged for 10 minutes at 12000rpm. The content discarded; around 250ul of 75%ethanol was added. The tube was centrifuged for 3 minutes at 80000rpm and the content discarded. Then finally, 60ul Distilled water was added, and the tube was stored at -80˚C until used.

Amplification of Cathepsin B3 from Immature Flukes

The RT-PCR primers targeting the Cathepsin B3 enzyme gene (cat-B3 mRNA) were used. Complete genome sequence of Fasciola Cathepsin B3 was obtained from the GenBank. The sequence of primers is as follows: CatB3F ATG AGT TGG TTG CTC ATA TTT GCT G and CatB3R TTA AGG TAA TCC GGC ATT AAT CCT G. Amplicon size of this gene is 1014bp. The RT-PCR reaction in a volume of 10.0 μl in total containing 2.6 μl RNAse free water, 2.0 ul M-MLV buffer, 1 ul dNTP, 1.0 ul of each forward and reverse primer, 0.2 ul enzyme mix (Taq + M-MLV reverse transcriptase), and 2.0 μl of total RNA as template (blood RNA extract) was performed. The profile that was followed a PCR run

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of 30 minutes at 45°C followed by 10 min at 95ºC. There were 35 amplification cycles, of which, there is denaturation of 30 seconds at 94°C, 1 minute primer-template annealing at 60°C, and polymerization for 1 minute at 72ºC. For 10 minutes, a final elongation step was put throughout at 72°C and stored at 12ºC infinitely. Each RT-PCR runs sterile double distilled water (DDW) as a negative control and cDNA of immature flukes as a positive control.

RT-LAMP assay

The designed LAMP primers for the target gene FgCatB3 expressed the Cathepsin B3 applying the software LAMP Designer 1.10. Table 1 shows the set of primers designed to be used in the study.

Table 1: Primer sequences of RT-LAMP used in this study (adapted from Aquino, 2017)

PRIMER NAME	SEQUENCE
F3	CCATCCACTCGATTCAACAA
B3	TACGCACAACAACTGACAA
FIP	CGAGCATCGAAAGATTCGGGTA + CGGAACACTCAACGACAA
BIP	TTCCGAGATTCGTGACCAATCC + TTATTGCCGAAGCAGAGC
FLoop	CTGATACGGAATACCTCACGG
BLoop	TAGTTCGTGTTGGGCTGTC

Preparation of the LAMP primer mix was done. From the 100 uM stock of every primer preparation, 2.5 ul was taken from each of the F3 and B3 primers, 10 ul from each of the FIP and BIP primers and 5 ul from each of the F-loop and B-loop primers. All these were mixed into one tube labeled as the primer mix.A 12.5-µL RT-LAMP mix was dispensed into each reaction tube. Each tube comprised of the following components with corresponding concentrations: 4.1 ul nuclease-free water, 0.9 uL of 100uM $MgSO_4$, 1.5 ul of 10x RT-LAMP buffer, 3 ul of 5M Betaine, 1 ul of 10mM dNTP mix, LAMP primer mix (0.5 ul) , 0.25 ul of 8 units/uL of Bst DNA polymerase and 0.25 ul of reverse transcriptase. For each RT-LAMP reagent mix, one µL of RNA extract of blood was added. It was incubated at different temperatures set at 60˚C and 62˚C for 90 minutes. One uL of 10x SYBR Green I fluorescent dye was added to each tube after completion of the reaction.

Dye and Fluorescence Test

A dye test is the color change of RT-LAMP assay results after the addition of 1µl SYBR Green I as seen by the naked eye. Green color indicates positive result while orange color means negative result. Subsequently, for the fluorescence test, RT-LAMP results were viewed under UV light. Positive results fluoresced while the negative did not. RT-LAMP results were confirmed using gel electrophoresis. Using the molecular-grade agarose gel (1.5%) with 1x Tris-Acetate-EDTA buffer and 1.8µl of Gel Red , 3µl of the RT-LAMP product was loaded in gel electrophoresis (Mupid-One) system at 100V for 30 minutes. Results were viewed under the UV illuminator. Positive results showed multiple ladder-

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like bands in the gel. The prevalence of infected snails were presented from the different barangays and by different RT-LAMP indicators used.

The formula for Positivity rate:

$$\text{Positivity rate} = \frac{\text{no. of positive}}{\text{Population size}} \text{ X } 100\%$$

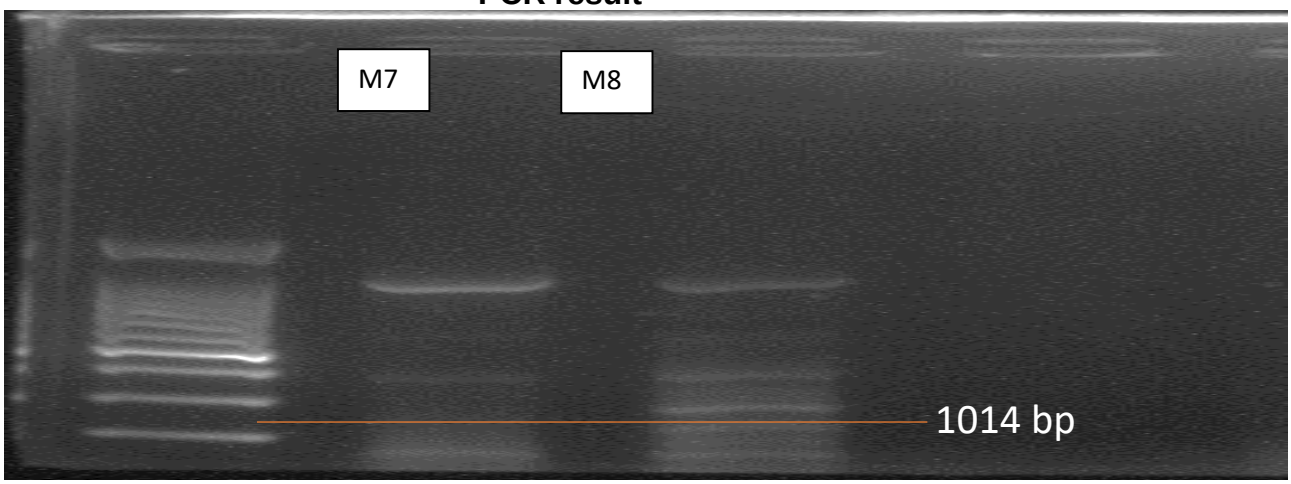
3) RESULTS AND DISCUSSION

According to Soulsby (1982), the intermediate host, snails commonly present in poorly drained land, irrigation tunnels, wet and muddy places of near grazing pasture, and drinking water. Lymnaea truncatula is commonly found in irrigation tunnels, small stagnant water bodies, and streams that move slowly. According to Soulsby (1982), the intermediate host, snails commonly present in poorly drained land, irrigation tunnels, wet and muddy places of near grazing pasture, and drinking water. The snail, Lymnaea truncatula, is commonly found in irrigation tunnels, small stagnant water bodies, and slowly moving streams.

RT-PCR

Among the eight juvenile flukes extracted for RNA, two were RT-PCR positive with amplified products having 1014 bp molecular weight. RT-LAMP assay also showed the expected ladder-like bands. Thus, this shows that the juvenile flukes extracted carry the F3CatB3 gene. Different studies confirmed the sensitivity of PCR to different parasites. DNA-based molecular tests, including PCR, can be used for the clinical detection of Fasciola spp. (Attlahet al., 2013) as cited by (Hulipas 2016). But based on the results, RT-PCR is not sensitive compared to RT-LAMP for detecting the gene cathepsin b3 in juvenile flukes.

Figure 2: Gel image of amplified F3CatB3 gene from two juvenile flukes using RT-PCR result



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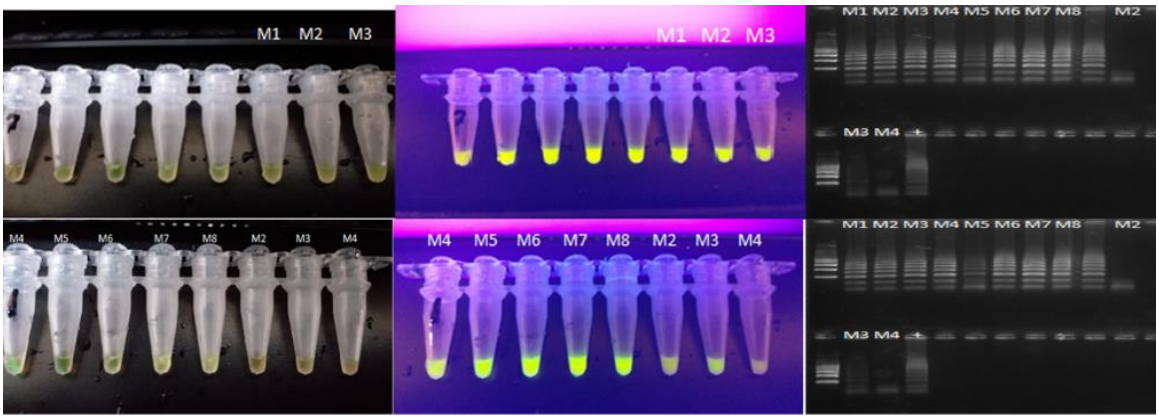
RT-LAMP Protocol Optimization

The researcher optimized eight marita samples by RT-LAMP after storage. Figure 3 shows the RT-LAMP protocol optomization confirmed by dye and fluorescence test and gel electrophoresis. The time for RT-LAMP amplification as shown by the dye, fluorescence, and gel image indicators and optimal reaction temperature was 62˚C for 90 minutes. Cong Cao of the University of Tennessee-Knoxville compared the RT-PCR and RT-LAMP in sensitivity of human norovirus. For extracted RNA, He found out that the RT-LAMP assay is more sensitive than the RT-PCR assay. In addition, (Li et al., 2002) confirmed that the LAMP assay is around 10^4 times sensitive than the conventional PCR assays. Hence, RT-LAMP assay has a great advantage in simplicity, rapidity, accuracy, cost-effectiveness, and higher amplification efficiency (Mori et al., 2009; Notomiet al., 2000) as cited by Aquino, 2017.

These previous findings on LAMP as a faster and easier test could imply that in this study, the RT-LAMP assay may also have a potential clinical application especially in endemic areas for detecting Fasciola species.



3A **3B** **3C**

Figure 3: RT-LAMP Assay results of DNA extracts from juvenile flukes. Figure 3A shows the dye test indicating the change in color from orange (-) to green (+) after the addition of SybrGreen dye. Figure 9Bshows the fluorescence test after viewing under UV light. Figure 3C shows the gel image after electrophoresis of RT-LAMP reactors where multiple ladder-like bands indicate presence of the FgCatB3 gene which expresses Cathepsin B3 in juvenile flukes.

Field Validation of Cathepsin B3 RT-LAMP Protocol from Blood Samples of Cattle and Swamp Buffalo

A total of 160 blood samples from cattle and swamp buffaloes were collected. Using RT-LAMP to detect the FgCatB3 gene, 44 blood samples were detected as positive or a prevalence of 27.5 %. This statement indicates that the gene from juvenile Fasciola

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(Marita stage) was present in both infected cattle and swamp buffaloes; hence, it could imply that they were harboring immature flukes. Table 2 presents the positivity rate of cattle and swamp buffaloes positive for Fasciola infection. Table 2 shows that more swamp buffaloes were positive for the cathepsin B3 gene than cattle. This data implies that more swamp buffaloes may be carrying juvenile Fasciola in their circulation. Buffaloes have higher prevalence than cattle in a study conducted by Molina et al. (2005). She also compared on her separate study the host and parasite relationship of cattle and swamp buffaloes and Fasciola gigantica , wherein she detailed the immune responses of cattle and buffaloes during the infection with F. gigantica. The different responses to F. gigantica infection indicate the host-parasite relationships of F. gigantica and large ruminants. This is associated to the diverse amount of resistance and resilience between the hosts to infection. In simple words, the infection of these two species to Fasciolosis depends on their immune responses.

Table 2: Positivity rate of Cathepsin B3 gene in blood of swamp buffaloes and cattle according to origin

PLACE OF ORIGIN	SWAMP BUFFALO			CATTLE		
	No. of examined	No. of LAMP positives	Positivity rate	No. of examined	No. of LAMP positives	Positivity rate
Baler	20	7	40%	20	3	15%
Dipaculao	20	6	25%	20	3	15%
Maria	20	6	30%	20	4	20%
San luis	20	6	35%	20	9	40%
TOTAL	80	25	32.5%	80	19	22.5%

Overall prevalence = 27.5% (44/160)

Table 3: Prevalence of infected blood samples based on age of cattle and swamp buffalo

PLACE OF ORIGIN	SWAMP BUFFALO			CATTLE		
	No. of LAMP positives			No. of LAMP positives		
	young	adult		young	adult	
Baler	2	5		2	1	
Dipaculao	5	1		2	1	
Maria	2	4		2	2	
San luis	4	2		1	8	
TOTAL	13	12		7	12	

Table 3 shows out of 44 blood samples, more caracalves are positive for the gene of cathepsin B3 than adults. On the other hand, in cattle, more adult cows/bulls are positive for the gene of cathepsin B3. Results could imply that the caracalves were continuously exposed to fresh metacercariae infection because they usually follow the adults who love to wallow in water where cercaria-shedding snails may abode compared to their calf

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counterparts. This result shows that the prevalence between the caracalves and adults did not differ remarkably. But based on a study conducted by Mamun et al. (2011), the age of the buffaloes had a significant effect on gastro-intestinal parasitic infection. Hence, they have included Fasciola gigantica among the nine parasites they've studied. Young buffaloes were more susceptible to infection than calves and adult buffaloes.

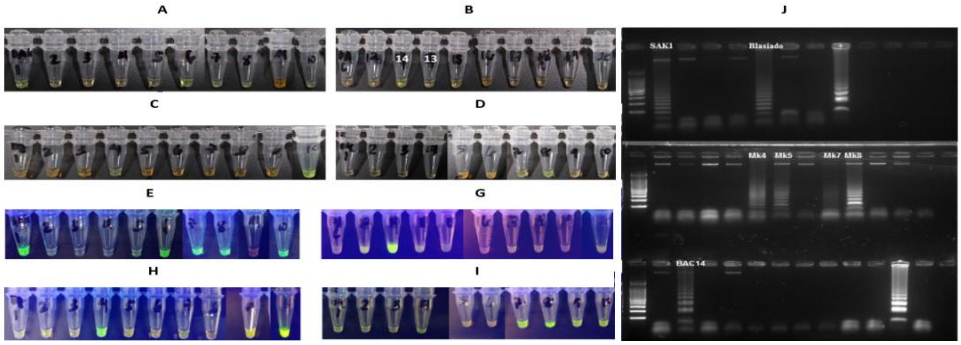

Fig 4: RT-LAMP Assay results of representative blood samples. Dye test indicating the change in color from orange (-) to green (+) after the addition of SYBR Green (Figures 4A - D). Figures 4E – I show the results of the fluorescence test viewed Under the UV light. Positive result shows green luminescence. Figure 4J shows the gel image after electrophoresis of RT-LAMP reactors. Multiple ladder-like bands indicate the presence of the amplifiedFgCatB3 gene.

As shown in Figure 4, after the addition of SYBR green, representative blood samples turned green, and these were confirmed consistently in the fluorescence test under UV light. The appearance of a white magnesium pyrophosphate precipitate or by color change on the addition of SYBR green I to the reaction mixture determine the presence or absence of DNA (Liang et al., 2009) as cited by Alvaran, 2016.Furthermore, RT-LAMP assay results were also confirmed using gel electrophoresis. The presence of multiple ladder-like bands is due to the stem-loops of DNAs accumulated during the cycling reaction (Notomiet al., 2000), as cited by Alvaran (2016). Blood samples were labeled according to municipalities and the age of cattle and buffaloes.

Table 4: Prevalence of blood samples based on Cathepsin B3 RT-LAMP indicators

Rt-lamp indicators	No. Of positive	No. Of negative	Total	Prevalence
Dye test	44	116	160	27.5%
Fluorescence test	44	116	160	27.5 %
Gel electrophoresis	44	116	160	27.5%

Out of the four (4) municipalities screened, all samples were later confirmed by gel electrophoresis. These were all rightfully extracted and showed consistent results throughout the different LAMP indicators: dye, fluorescence, and gel electrophoresis (see table 4). In diagnosing helminth infection there are different traditional parasitological

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process, serological tests, and PCR-based assays used but they are time-wasting and expensive and sometimes the results are inaccurate. In detecting helminths, a sensitive, simple and rapid method, Loop-mediated isothermal amplification (LAMP) assay was developed (Deng et al., 2019). Animal owners were interviewed to know the practices they apply in providing water and forage to their animals.

Tables 5 and 6 shows the percentage distribution of infected cattle and swamp buffalo that were RT-LAMP positive for the FgCatB3 gene in their blood according to how their owners provided them drinking water. Table 5 shows that 37 % (13/35) of cattle led to drinking in rivers or streams were positive as compared to those provided by the farmers with drinking water (27%; 12/45). Table 6 shows that 32 % (18/56) of swamp buffaloes that drank water in rivers or streams were positive compared to those provided by the farmers with drinking water (17%; 4/24). This result confirms the statement of (Payne 1990), as cited by Zeleke (2013) that the infestation of fasciolosis is usually associated with grazing and drinking ways in places where snails are found.

Table 5: Distribution of infected cattle using RT-LAMP according to how they access their drinking water

Access to drinking water	No. Examined	Infected cattle	
		Number	Percent (%)
Led to rivers or streams	35	13	37%
Provided by owner	45	12	27%
TOTAL	80	25	31%

Table 6: Distribution of infected buffaloes using RT-LAMP according to how they access their drinking water

Access to drinking water	No. Examined	Infected buffaloes	
		Number	Percent (%)
Led to rivers or streams	56	18	32%
Provided by owner	24	4	17%
TOTAL	80	22	28%

1) infestation with fasciolosis is usually associated with grazing wetland and drinking from the snail infesting watering
2) places (Payne, 1990
3) infestation with fasciolosis is usually associated with grazing wetland and drinking from the snail infesting watering
4) places (Payne, 1990
5) infestation with fasciolosis is usually associated with grazing wetland and drinking from the snail infesting watering

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6) places (Payne, 1990
7) infestation with fasciolosis is usually associated with grazing wetland and drinking from the snail infesting watering places (Payne, 1990

Tables 7 and 8 show the distribution of infected cattle and swamp buffaloes according to how they were given forage. Table 7 shows that 27% (7/26) of cattle that were allowed to graze in communal pastures for their forage were positive in RT-LAMP as compared to those that were either tethered in pasture and by “cut and carry” in times of severe weather (22%;12/54). As for buffaloes, 26% (9/35) swamp buffaloes were positive when allowed to graze in a communal pasture compared to buffaloes that were tethered and provided forage through "cut and carry" during inclement weather. Livestock owners interviewed for both grazing and providing feed said they do “cut and carry” when there are storms and if their area is flooded.

According to Soulsby 1982, as cited by Zeleke 2013 the intermediate hosts, snails, are commonly present in poor, drained land, irrigation tunnels, and muddy places near grazing pasture and drinking water. This is why Eknuwife, 2006 suggested that cattle grazing should be highly restricted to areas of lesser snail infected sites. The rangeland systems (Artificial pasture land) seem to be the panacea to fascioliasis.

Table 7: Distribution of infected cattle using RT-LAMP according to how they access forage

Access to forage	No. Examined	Infected cattle	
		Number	Percent (%)
Communal grazing	26	7	27%
Tethered and or “cut and carry”	54	12	22%
TOTAL	80	19	24%

Table 8: Distribution of infected buffaloes using RT-LAMP according to how they access forage

Access to forage	No. Examined	Swamp buffaloes	
		Number	Percent (%)
Communal grazing	35	9	26%
Tethered and or “cut and carry”	45	11	24%
TOTAL	80	20	25%

Summary

RT-LAMP optimization to detect the gene expressing Cathepsin B3 in juvenile Fasciola spp. of an infected liver showed optimal temperature and reaction time for amplification at 62C for 90 minutes as confirmed in the dye, fluorescence test, gel electrophoresis, and RT-PCR. Field validation of the RT-LAMP protocol was done on blood samples of swamp buffaloes and cattle to detect the gene of Cathepsin B3. RT-LAMP prevalence

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was 27.5 %, with more caracalves showing optimism than their calf counterparts.

Conclusion

Prepatent detection of fasciolosis in large ruminants can be possible using the optimized RT-LAMP that can amplify that gene expressing the cathepsin B3 enzyme, which is secreted exclusively by juvenile flukes. Blood samples can be examined for prepatent detection. Hence, strategic deworming can be implemented before the flukes reach the adult stage and obstruct the bile ducts leading to death.

Recommendations: It is recommended that the laboratory workflow be translated into a kit so that prepatent detection of fasciolosis for strategic deworming can be done.

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