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Development of A Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Human *Fasciola* in Vietnam

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ABSTRACT

Fasciola infestation in humans is known to have species Fasciola gigantica and Fasciola hepatica. These species also cause diseases mainly of ungulates such as buffaloes, cows, and sheep.. The disease has also been reported in 75 countries around the world. The diagnosis of disease in humans and animals is mainly based on egg and fluke morphology, or immunology and clinical symptoms. These techniques are often late and confusing, while molecular biology is costly, especially in undeveloped countries like Vietnam. Developing a fast and accurate detection technique is very important and necessary. In this study, we developed the LAMP technique to quickly and accurately detect Fasciola spp. from different kinds of samples such as feces, tissue human samples with a sensitivity and specificity of over 95%. This method will help increase sensitivity, reduce engineering time, and lower cost, this is considered a superior advantage over other diagnostic techniques. The primer set was designed for founding on ITS1-2 gene. A Positive reaction was visualized with the naked eye when using the color indicator, Malachite Green.

Keywords

Fasciola spp, Loop-mediated isothermal amplification (LAMP).

Background

Species of *Fasciola* cause disease known as fasciolosis (or fascioliasis) in domestic animals (cattle, sheep, goats, donkeys), wild mammals (rabbits, beaver, deers, water buffalo, and camels), and humans [1,4,5,7]. Reported by the World Health Organization (WHO), the disease is currently spreading in 75 countries and regions around the world [29]. WHO considers the disease a health issue that needs attention in public health programs and has been ranked by many countries as an important position in health strategy and policy. Epidemiological characteristics, the way of transmission, distribution of liver fluke depends on the distribution of intermediate hosts of snails of the genus Lymnaea and climatic conditions, so the prevalence of the disease varies between nations [7]. Vietnam is located in the tropics, with favorable natural and social conditions for parasites such as *Fasciola spp*. to develop, causing greatly affect the health of the people. In Vietnam, fascioliasis has been reported as zoonotic, the most common being *Fasciola gigantica*. In the previous stage, the infection of the liver fluke was large in a low person, mainly reporting cases in the hospital. Recently, the number of *F. gigantica* liver fluke infections has been recorded to increase rapidly and distributed in many places nationwide. According to reports from the National Institute of Malariology, Parasitology and Entomology (NIMPE), patients with fascioliasis have been discovered in many provinces and cities nationwide. The disease is found mainly in patients over 15 years of age and tends to increase in recent years as in 2011, there were about 10,407 cases distributed in 53 provinces and cities nationwide were treated. And In 2019, there were 12,309 cases.

Developing a fast and accurate detection technique is very important and necessary. Currently, the diagnosis of fasciolosis is confirmed by the observation of parasite eggs in the feces, but the disadvantage of this method is low sensitivity (about 30-40%), specificity depends much on the experience of technicians. Several immunological techniques have also been developed and used for the detection of antibodies developed in the host against infection. These techniques include ELISA and Western blot methods [9,11,12,13,14,19,22,24]. These methods are more sensitive but maybe have false negatives caused by host antibodies. Molecular methods were developed to increase the sensitivity and specificity of conventional diagnosis [2,5,6,7,17,25,26]. PCR and qPCR techniques have high sensitivity and specificity but require expensive equipment and difficult to apply in the field. Loop-mediated isothermal amplification (LAMP) is an outstanding molecular method that is widely used in diagnosis. This technique has the same specificity and sensitivity as PCR, but the amplification process is carried out at a constant temperature [20,21]. In this paper, we report our research on the validity of the LAMP technique for the detection of *Fasciola spp* from specimens.

Methods

Samples

Samples used for positive and negative control purposes: For this study, the adult worms (*Fasciola hepatica, Fasciola gigantica, Clonorchis sinensis, Opisthorchis iverrine, Haplorchis taichui, Haplorchis pumilio, Gnathostoma spinigerum*), were obtained from Parasitology Department, National Institute of Malariology, Parasitology and Entomology (NIMPE), Vietnam. DNA from *Fasciola* spp. was used as positive control and DNA from other helminths was also extracted to determine the specificity.

- Fecal samples were collected from the Clinical Department, NIMPE, Vietnam, and Quang Nam, Binh Dinh provinces, Vietnam.

DNA extraction

Total DNA was extracted from aldult worm and feces by QIAamp DNA micro kit and QIAamp DNA stool mini kit of Qiagen (Germany). The extraction procedure is performed according to the manufacturer's instructions.

LAMP primer design

Primers used for the LAMP assay were designed based on a highly conserved region of *Fasciola spp*. Genome. Genbank sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (table 1) including the internal transcribed spacer 2 (ITS2) region were tested in silico through BLAST searches and alignment analysis using the Clutal W module of MEGA 7 software. A 461 base pairs (bp) consensus sequence was seclected for the design of specific primers using Primer Explorer V5 (http://primerexplorer.jp/lampv5e/index.html). Two sets of primers comprising two outer (F3 and B3) and two inners (FIP and BIP) were selected. FIP contained the F1c (complementary to F1) and F2 sequences. BIP contained the B1c (Complementary to B1) and B2 sequences.

Table 1: Genbank accession numbers for the different *Fasciola spp*. isolates included in the alignment.

No	Isolates	Genbank accession number
1	*Fasciola sp.*	JF708041.1
2	*Fasciola sp.*	KF543341.1
3	*Fasciola sp.*	MK330625.1
4	*Fasciola sp.*	MN310035.1
5	*Fasciola gigantica*	MN970010.1
6	*Fasciola gigantica*	MN784632.1
7	*Fasciola gigantica*	MN608173.1
8	*Fasciola gigantica*	MK330622.1
9	*Fasciola gigantica*	KX198631.1
10	*Fasciola gigantica*	KP760871.1
11	*Fasciola gigantica*	KJ789345.1
12	*Fasciola gigantica*	KF543340.1
13	*Fasciola gigantica*	JF432073.1
14	*Fasciola gigantica*	HM746788.1
15	*Fasciola gigantica*	JF496715.1
16	*Fasciola gigantica*	JN828955.1
17	*Fasciola gigantica*	AJ853848.2
18	*Fasciola gigantica*	AM900371.1
19	*Fasciola hepatica*	MN784625.1
20	*Fasciola hepatica*	MN559388.1
21	*Fasciola hepatica*	MK212150.1
22	*Fasciola hepatica*	MG569981.1
23	*Fasciola hepatica*	KX856340.1
24	*Fasciola hepatica*	KU555843.1
25	*Fasciola hepatica*	KX198630.1
26	*Fasciola hepatica*	KJ789365.1
27	*Fasciola hepatica*	JF432078.1
28	*Fasciola hepatica*	HM746786.1
29	*Fasciola hepatica*	JF496716.1
30	*Fasciola hepatica*	JN828954.1
31	*Fasciola hepatica*	GQ231547.1
32	*Fasciola hepatica*	AM709498.1

Optimization of the assay

PCR assay

The outer primers of the LAMP primer designed in this study (the F3 and B3 primers) were tested for their specificity in PCR. DNA from adult helminths which comprised of *Fasciola hepatica, Fasciola gigantica, Clonorchis sinensis, Opisthorchis iverrine, Haplorchis taichui, Haplorchis pumilio* were used as the DNA templates for the specificity test. A standard PCR, using 0,5µM of primers F3 and B3 and 2 µl DNA, was carried out in the total volume of 20µl using the HotstarTaq Plus Master Mix kit (Qiagen, Germany). The annealing temperature is 60^0C for 30 seconds. PCR products were analyzed by electrophoresis in 2% agarose TBE. All reactions were carried out in triplicate.

LAMP assay

These was set up testing different temperatures, $MgSO_4$ concentrations, reaction time, visually detected dye concentrations. LAMP reaction mixtures (25µl) contained 1x Isothermal Amplification buffer (New England Biolabs, UK), $MgSO_4$ (4, 6 or 8 mM) (New England Biolabs, UK), 1,4mM of each dNTPs (Qiagen, Germany), 5pmol of each F3 and B3 primers, 40pmol

of each FIB and BIP primers, 20pmol of each LF and LB primers, and 8U of Bst 2.0 DNA polymerase (New England Biolabs, UK) with 5µl of DNA. Different temperatures were tested using a thermocycler (PCR gradient Nesux GX1, Eppendorf) set from 56oC to 65oC for 40 and 60 minutes and then 80oC for 5 minutes. Amplifications were visually detected by adding Malachite Green (MG) at 0,012%, 0,008%, 0,004% and 0,001%. Light blue was observed in successful LAMP reactions; colorless in negative reactions. The LAMP reaction products were also analyzed by electrophoresis in 2% agarose TBE. All experiments were done in triplicate.

The limit of detection (LOD) of the LAMP assay was determined by two periods. First using a ten-fold serial dilution of recombinant plasmid DNA containing the *Fasciola spp*. ITS 2 gene (from 10^{-6} ng/µl to 10^{-11} ng/µl) in comparison to the LAMP-2.0% AGE and standard LAMP-turbidity assay. Each experiment was done in triplicate. The lowest concentration at which a LAMP post-reactive product is always present is the primary threshold of detection to look for. Period two: Perform threshold detection with 95% confidence (LOD95%) by LAMP reaction with the diluted recombinant plasmid concentration range asymptotically to the concentration of the primary detection threshold of period 1. Beside, Serial 10 fold dilutions from DNA of adult worms *Fasciola spp*. were also prepared to range from 10^{-1} ng/µl to 10^{-6}ng/µl to determine the sensitivity.

Preparation of *Fasciola spp*. plasmid DNA template

The specific PCR primers for cloning were designed based on a highly specific region of the ITS2 genes of *Fasciola* spp. Then, genomic DNA was subsequently amplified by PCR, which produced a product 461 bp in size. Once the PCR was completed, the PCR product was purified and cloned into the pUC19 vector (Invitrogen, USA). Concentrations of recombinant plasmid DNA were measured with a Nanophotometer (IMPLEN, Germany), and corresponding copy numbers were calculated as mole multiplies of Avogadro's number using an online program (http://cels.uri.edu/gsc/cndna.html), following the formula: The number of copies = Amount × 6.022 × 10^{23}/length × 10^{9} × 650.

Clinical sample tests

A total of 110 fecal samples were collected from Binh Dinh and Quang Nam provinces, Vietnam, and 20 fecal samples from the Clinical Department, NIMPE, Hanoi, Vietnam. All these clinical samples were evaluated by LAMP assay comparing with qPCR as a gold standard test. The percentage of sensitivity, specificity was calculated in the format of a 2×2 cross-tabulation table.

LAMP method	Gold standard test†	
	Positive	Negative
Positive	TP	FP
Negative	FN	TN
Total	TP + FN	FP + TN

† TP = true positive. , FP = false positive, TN = true negative, FN = false negative.

$$\text{Sensitivity}\,(\%) = \frac{TP}{TN} \times 100$$

$$\text{Specipicity}\,(\%) = \frac{}{FP + TN} \times 100$$

Results

Using BLAST comparisons of LAMP primers showed a homology of 100% with *F.hepatica* and *F.gigantica*. The PCR specificity test using F3 and B3 primers of LAMP Primer amplified DNA from *F. hepatica, F.gigantica* with an amplicon length of around 218 bp (visualized by gel electrophoresis and UV detection). No amplification of DNA from any of the other helminths DNAs used in the test was observed (Figure 1).

Table 2: List of primers used in this study.

Primer name	Sequence
F3	GGTTGGACTGATAACCTGG
B3	CTTTTTGGGCGTCGTGAT
FIP	TGCGCTCTTCATCGACACAC-TTGACCATACGTACAACTCT
BIP	ACTGCTTTGAACATCGACATCTTGA-TTTATAAGCCGACCCTCG
LF	AGCCGAGTGATCCACCG
LB	TTAGCCTGTGGCCACGC

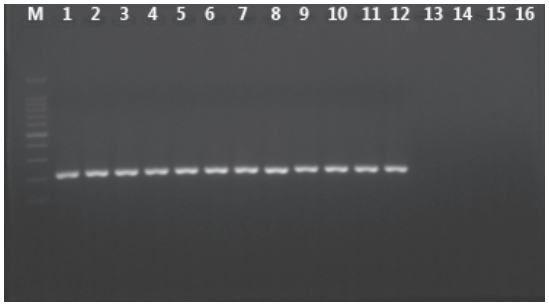

Figure 1: PCR products using F3-B3 primers of *Fasciola* spp.: 1-6: *F.hepatica*; 7-12: *F.gigantica*; 13: *Clonorchis sinensis*; 14: *Opisthorchis viverrini*; 15: *Haplorchis taichui;* 16: *Haplorchis pumilio.*

The data revealed that the reaction temperatures at 63°C and the concentration of $MgSO_4$ 8mM had the highest LAMP amplification efficiency (Figure 2). Hence the intermediate temperature at 63°C, and the concentration of $MgSO_4$ at 8mM were chosen to perform every LAMP test in this study.

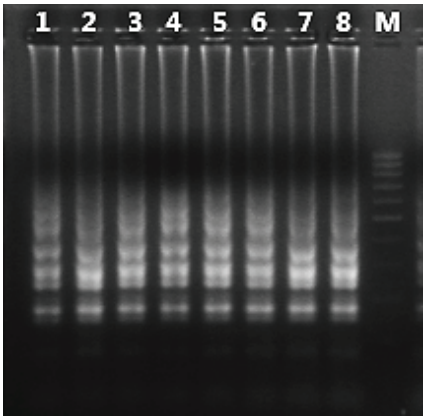

Figure 2: LAMP product at 63°C and $MgSO_4$ 8mM of *Fasciola*.

The optimum concentration of MG dye indicated that 0.004% MG was suitable for discrimination of the results as light blue and colorless in positive and negative reactions, respectively (Fig. 3).

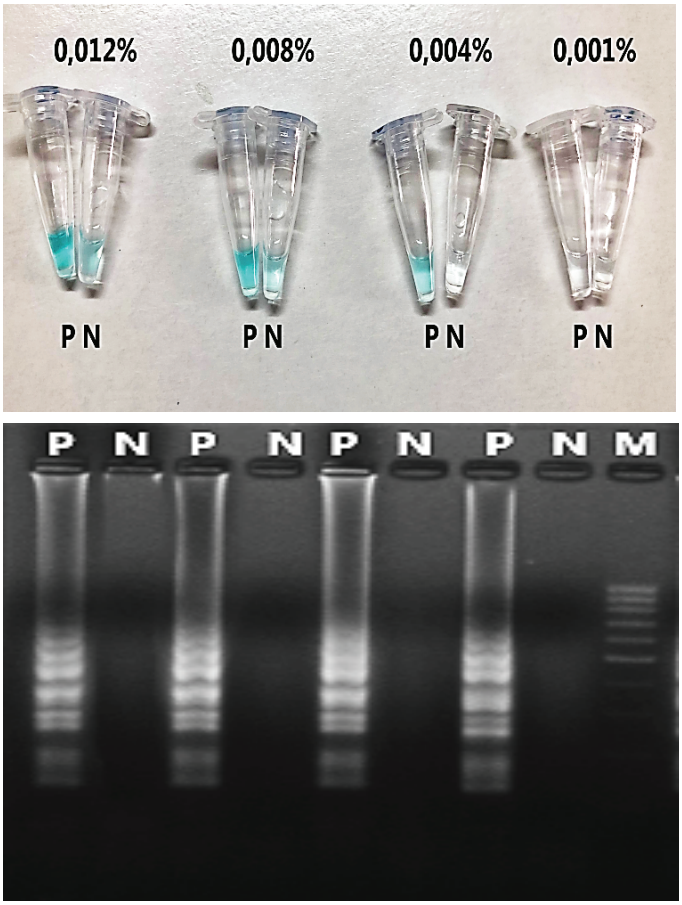

Figure 3: MG concentration tests at 0.012%, 0.008%, 0.004% and 0.001% at 63 °C for 60 min. P and N represent positive and negative reactions, respectively.

Table 3: Results of the detection threshold of LAMP primers.

Concentrate of DNA copies (ng/µl)	Result		
	1st repeat	2nd repeat	3rd repeat
10^{-6}	(+)	(+)	(+)
10^{-7}	(+)	(+)	(+)
10^{-8}	(+)	(+)	(+)
10^{-9}	**(+)**	**(+)**	**(+)**
10^{-10}	(-)	(-)	(-)
10^{-11}	(-)	(-)	(-)
Neg1	(-)	(-)	(-)
Neg2	(-)	(-)	(-)

The primary detection limits of the LAMP were 2.94×10^{-1} copies/µl DNA (Table 3; fig. 4). The amplification products were visualized on an agarose gel as a ladder of multiple bands (Figure 4). The limit of detection of LAMP using aldult worm DNA was 10^{-6}ng/µl.

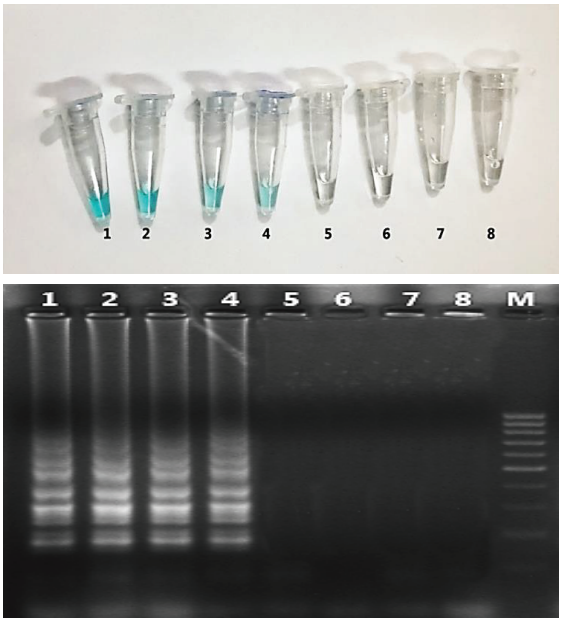

Figure 4: Detection limit of LAMP-MG assay and LAMP-2.0% AGE of *Fasciola*: 1-6: 10^{-6} to 10^{-11} ng/µl; 7-8: negative control.

Table 4: Investigation of the threshold of LAMP for detection of fasciolosis.

Concentrate of DNA copies (ng/µl)	Number of copies (Copies/µl)	Times repeat	Time Positive	Positive rate (%)
$1x10^{-6}$	$2.94x10^{2}$	12	12	100.00
$1x10^{-7}$	$2.94x10^{1}$	12	12	100.00
$1x10^{-8}$	$2.94x10^{0}$	12	12	100.00
$1x10^{-9}$	$2.94x10^{-1}$	12	12	100.00
$7.5x\ 10^{-10}$	$2.21\ x10^{-1}$	12	11	91.67
$5x10^{-10}$	$1.47x10^{-1}$	12	8	66.67
$2.5x10^{-10}$	$7.35\ x10^{-2}$	12	6	50.00
$1.25x10^{-10}$	$3.68x10^{-2}$	12	4	33.33
$1x10^{-10}$	$2.94x10^{-2}$	12	1	8.33
$6.25\ x10^{-11}$	$1.84\ x\ 10^{-2}$	12	0	0.00
0	0	12	0	0.00

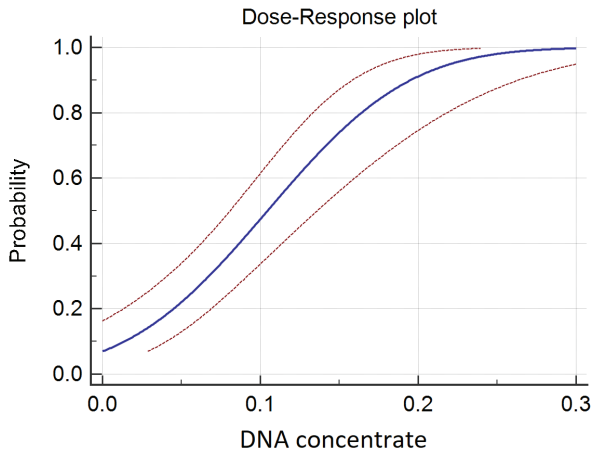

Figure 5: Graph showing LOD95% of LAMP kit for fasciolosis diagnosis.

From Table 4 it shows that LOD95% of primer is 2.21x10-1 number of gene copies / µl (95% CI: 1.79x10-1 gene copy / µl to 3.01x10-1 gene copy / µl). This is a good threshold of detection, allowing the detection of the pathogen at low density.

The validity of the LAMP-MG assay was calculated against qPCR as a gold standard test. The data demonstrated that the sensitivity = 96.67%, specificity = 97.0%.

Table 5: Sensitivity and specificity of LAMP detect *Fasciola* spp.

	qPCR	
LAMP	Positive	Negative
Positive	29	3
Negative	1	97
Total	30	100
	Se: 96.67% (95% CI: 82.78% - 99.92%)	Sp: 97.00% (95% CI: 91.48% to 99.38%)

Discussion

With an increasing number of fasciolosis cases in Vietnam since 2011 as reported by the Department of Parasitology, NIMPE, Fasciolosis has become an emerging infectious disease that can be seriously affected by human health. Thus, the early detection of *Fasciola* spp. infection is required and essential for the people in endemic areas. Herein, a LAMP-MG colorimetric assay has been developed as a rapid, sensitive, and specific tool for the detection of *Fasciola* based on the ITS2 gene due to its uniqueness in individual-specific sequence genus. We also tested our assay specificity with *Clonorchis sinensis, Opisthorchis viverrini, Haplorchis taichui, Haplorchis pumilio* with satisfying results. However, our assay did not test other helminths. The nucleotide sequence analysis using the BLAST showed that no helminths were related to our primer sequences (data not shown).

Numerous studies have demonstrated that the LAMP technique is a highly specific, sensitive, short reaction time and allows to check amplification products simply by observing turbidity, by drug fluorescent dye or pH indicator dye. Each testing method has certain advantages and disadvantages. Although the white pyrophosphate generated by the LAMP reaction can be detected with the naked eye, it has a short precision (about 5-10 seconds) after taking the sample out of the incubator.

Therefore, this type of detection may require a real-time turbidity meter for more accurate conclusions. Using fluorescent dyes such as calcein or SYBR Green I is expensive and requires a UV light system to read the results. Moreover, calcein can combine with Mg^{2+} ions to inhibit DNA polymerase activity and reduce the general sensitivity of the test. The addition of SYBR Green I after the reaction may increase the contamination of DNA, leading to erroneous results.

Recently, Malachite Green (MG) color indicator has been successfully used as a pH-sensitive indicator to detect LAMP products. The color of Malachite Green (cationic form) changes depending on the pH of the solution and hence the pH. 2 represents yellow, pH = 3-9 is blue, and pH> 10 is colorless. Malachit Green has an absorption wavelength of about 621 nm. In this LAMP-MG assay, positive and negative samples visible to the naked eye often distinguish between changes in blue and colorless tones, respectively.

Using of MG as a pH-sensitive indicator dye for visual end-point assessment of LAMP products in various infections caused by bacteria [10] and protozoa [15,23,27] have been reported. The effect of MG in LAMP buffer prior to reaction does not affect the Bst DNA polymerase activity and also eliminates the risk of inter-sample contamination. The advantages of LAMP-MG assays can improve and overcome limitations from other LAMP findings as mentioned above. This study used MG with a stable optimum concentration of 0.004%, making positive and negative perfectly differentiated. While, the higher the concentration of MG leads to increasing false positives, the lower the concentration of MG will not distinguish between positive samples and negative samples.

The blue color in the positive tubes remains in color after the reaction for up to 6 weeks, at room temperature. Time after 6 weeks was not investigated in this study. The sensitivity of the LAMP assay 10^{-6}ng/µl which similar results were described by Martinez et al. [18] and Ai et al. [3], who reported a limit of detection of $1x10^{-3}$ng and 10^{-5}ng. The sensitivity and specificity of LAMP assay were evaluated with 130 clinical samples and used qPCR as a gold standard. The sensitivity and specificity of the new LAMP technique developed through the tests are all over 95%, indicating that the kit in this study has high sensitivity and specificity such as PCR and qPCR... PCR technique has high sensitivity and specificity but requires expensive equipment, difficult to apply in the field, so LAMP-MG has the same sensitivity and specificity as PCR. It does not require expensive equipment as the preferred direction and is increasingly applied in the world.

Conclusion

LAMP could be a reliable, convenient, and possible substitute for molecular diagnosis of *Fasciola* infection in the field. Low cost and high simplicity make LAMP a potential useful tool for diagnosing not only local health facilities but also the community.

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Commitment

This article is not in dispute about the interests of authors and other research groups in the same field.

References

1. Ahmadi NA, Meshkehkar M. Prevalence and long term trend of liver fluke infections in sheep, goats, and cattle slaughtered in Khuzestan, southwestern Iran. Journal of Paramedical Sciences. 2010; 1: 26-31.
2. Ai L, Chen MX, Alasaad S, et al. Genetic characterization, species differentiation, and detection of *Fasciola spp.* by molecular approaches. Parasites & Vectors. 2011; 4: 101.
3. Ai L, Li C, Elsheikha HM, et al. Rapid identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* by a loop-mediated isothermal amplification (LAMP) assay. Veterinary Parasitology. 2010; 174: 228-233.
4. Aksoy DY, Kerimoglu Ü, Oto A, et al. *Fasciola hepatica* infection: Clinical and computerized tomographic findings of ten patients, Turkish Journal of Gastroenterology. 2006; 17: 40-45.
5. Alasaad S, Soriguer RC, Abu-Madi M, et al. A TaqMan real-time PCR-based assay for the identification of *Fasciola spp*. Veterinary Parasitology. 2011; 179: 266-271.
6. Choe SE, Nguyen TTD, Kang TG, et al. Genetic analysis of *Fasciola* isolates from cattle in Korea based on second internal transcribed spacer (ITS-2) sequence of nuclear ribosomal DNA. Parasitology Research. 2011; 109: 833-839.
7. Cucher MA, Carnevale S, Prepelitchi l, et al. PCR diagnosis of *Fasciola hepatica* in field-collected Lymnaea columella and Lymnaea viatrix snails. Veterinary Parasitology. 2006; 137: 74-82.
8. Dusak A, Onur M, Cicek M, et al. Radiological imaging features of *Fasciola hepatica* infection- A Pictorial Review. Journal of Clinical Imaging Science. 2012; 2: 2.
9. Espino AM, Finlay CM. Sandwich enzyme-linked immunosorbent assay for detection of excretory-secretory antigens in humans with fascioliasis. J Clin Microbiol. 1994; 32: 190.
10. Farnia P, Masjedi MR, Mohammadi F, et al. Colorimetric detection of multidrug-resistant or extensively drug-resistant tuberculosis by use of malachite green indicator dye. J Clin Microbiol. 2008; 46: 796-799.
11. Flanagan AM, Edgar HWJ, Forster F, et al. Standardisation of a coproantigen reduction test (CRT) protocol for the diagnosis of resistance to triclabendazole in *Fasciola hepatica*. Veterinary Parasitology. 2011; 176: 34-42.
12. Flanagan A, Edgar HWJ, Gordon A, et al. Comparison of two assays, a fecal egg count reduction test (FECRT) and a coproantigen reduction test (CRT), for the diagnosis of resistance to triclabendazole in *Fasciola hepatica* in sheep. Veterinary parasitology. 2011; 176: 170-176.
13. Hassan MM, Saad M, Hegab MH, et al. Evaluation of circulating *Fasciola* antigens in specific diagnosis of fascioliasis. J Egypt Soc Parasitol. 2001; 31: 271.
14. Kajugu PE, Hanna REB, Edgar HW, et al. *Fasciola hepatica*: Specificity of a coproantigen ELISA test for diagnosis of fasciolosis in fecal samples from cattle and sheep concurrently infected with gastrointestinal nematodes, coccidians and/or rumen flukes (paramphistomes), under field conditions. Veterinary Parasitology. 2015; 212: 181-187.
15. Lucchi NW, Ljolje D, Silva-Flannery L, et al. Use of malachite green-loop mediated isothermal amplification for detection of Plasmodium spp. parasites. PLoS One. 2016; 11: e0151437.
16. Mahanty S, Maclean JD, Cross JH. Liver, Lung, and Intestinal Fluke Infections. Infectious Disease Advisor. 2011; 854.
17. Martínez-Pérez JM, Robles-Pérez D, Rojo-Vázquez FA, et al. Comparison of three different techniques to diagnose *Fasciola hepatica* infection in experimentally and naturally infected sheep. Veterinary Parasitology. 2012; 190: 80-86.
18. Martínez-Valladares M, Rojo-Vázquez FA. Loop-mediated isothermal amplification (LAMP) assay for the diagnosis of fasciolosis in sheep and its application under field conditions. Parasites & Vectors. 2016; 9.
19. Mezo M, González-Warleta M, Carro C, et al. An ultrasensitive capture elisa for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). Journal of parasitology. 2004; 90: 845-852.
20. Notomi T, Mori Y, Tomita N, et al. Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. Journal of Microbiology. 2015; 53: 1-5.
21. Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. Nucleic acids research. 2000; 28: e63-e63.
22. Novobilský A, Averpil HB, Höglund J. The field evaluation of albendazole and triclabendazole efficacy against *Fasciola hepatica* by coproantigen ELISA in naturally infected sheep. Veterinary Parasitology. 2012; 190: 272-276.
23. Nzelu CO, Cáceres AG, Guerrero-Quincho S, et al. A rapid molecular diagnosis of cutaneous leishmaniasis by colorimetric malachite green-loop-mediated isothermal amplification (LAMP) combined with an FTA card as a direct sampling tool. Acta Trop. 2016; 153: 116-119.
24. Palmer D, Lyon J, Palmer M, et al. Evaluation of a copro-antigen ELISA to detect *Fasciola hepatica* infection in sheep, cattle and horses: Production Animals. Australian Veterinary Journal. 2014; 92: 357-361.
25. Robles-Pérez D, Martínez-Pérez JM, Rojo-Vázquez FA, et al. The diagnosis of fasciolosis in faeces of sheep by means of a PCR and its application in the detection of anthelmintic resistance in sheep flocks naturally infected. Vet Parasitol. 2013; 197: 277-282.
26. Robles-Pérez, D, Martínez-Pérez JM, Rojo-Vázquez FA, et al. The diagnosis of fasciolosis in feces of sheep by means of a PCR and its application in the detection of anthelmintic resistance in sheep flocks naturally infected. Veterinary Parasitology. 2013; 197: 277-282.
27. Sarunya Tedlongthong, Nareerat Viseshakul, Hirotomo Kato, et al. Development of loop-mediated isothermal amplification

(LAMP) assay combined with malachite green as a rapid screening test for Candidatus Mycoplasma haemominutum infection in cats. ScienceAsia. 2017; 43: 354-361.

28. Weiss JB. DNA probes and PCR for diagnosis of parasitic infections. Clinical Microbiology Reviews. 1995; 8: 113-130.
29. https://www.who.int/foodborne_trematode_infections/fascioliasis/fascioliasis_epidemiology/en/