VOLUME-7, ISSUE-11, NOVEMBER-2018 • PRINT ISSN No 2277 - 8160

Original Research Paper

Medicine

APPLICATION OF REALTIME PCR AND ENVIRONMENTAL DNA ANALYSIS FOR DETECTING MINUTE FLUKES

Tran Thanh Duong	National Institute of Malagiology Parasitology and Entomology
Nguyen Thu Huong*	National Institute of Malagiology Parasitology and Entomology *Corresponding Author
Nguyen Thi Hong Ngoc	National Institute of Malagiology Parasitology and Entomology
Nguyen Thi Thanh Huyen	National Institute for Food Control
Nguyen Thi Huong Binh	National Institute of Malagiology Parasitology and Entomology

ABSTRACT Therefore, in this study we sought to develop a novel species-specific real-time PCR assay for detecting *C. sennisis* and or *O. viverrini* using environmental DNA (eDNA). The diagnostic sensitivity of the newly developed real-time PCR assay was similar to that of the traditional PCR assay for 50 fecal samples collected which were positive for small fluke egg were selected for adult and egg flukes in sedimentation feces. The efficacy of eDNA analysis and its applicability in the field were tested using a total of 120 environmental water samples collected from Bac Giang and Binh Dinh provinces in 2017. The results showed that *C. sennisis* and *O. viverrini* DNA was detected in 11 (22.0%) and 7 (14.0%) samples of sedimentation feces by real-time PCR. There were two of this pathogen eDNA in 2 (1.6%) and 4 (3.3%) water samples. The application of eDNA analysis would use the identification of fishborn-trematode endemic hotspots and contribute to the ecological control of closisenisis and opisthorchiasis, and this strategy can be applied to other eukaryotic environmental water pathogens.

KEYWORDS : Fishborn-trematode, real-time PCR, environmental DNA

1. BACKGROUND

Fish-borne trematodiasis is an infection characterized by minute flukes (MFs) that can cause chronic diseases and result in a neglected public health issue in Southeast Asia. Specifically, the liver fluke Opisthorchis viverrini is a common source of infection in Cambodia, Laos, Thailand, and Vietnam, whereas Clonorchis sinensis is distributed mainly in China, Korea, Taiwan, and Vietnam [5]. The zoonotic minute intestinal flukes species that reproduce small-sized eggs so far known in Vietnam include 7 species, namely, *Clonorchis sinensis*, *Opisthorchis viverrini*, *Haplorchis pumilio*, *H. taichui*, *H. yokogawai*, *Stellanchatmus falcatus* and *Echinostoma japonicus* [21]. Meat of reptiles, amphibians and fish can be infected with a variety of parasites, including trematodes (*Opisthorchis* spp., *Clonorchis sinensis*, and minute intestinal flukes)[10]. Infection with these food-borne parasites is prevalent in areas where uncooked cyprinoid fish are a staple of the diet. Due to poor sanitation practices and inadequate sewerage infrastructure, people infected with minute intestinal flukes pass parasite eggs in their faeces into natural water reservoirs, where the parasite eggs are eaten by intermediate host snails, for example, aquatic snails of the genus Bithynia, the first intermediate host of *O. viverrini*. After hatching, free swimming parasites, called cercariae, are released from the infected snails. Cercariae then locate their next intermediate host, cyprinoid fishes, encyst in the fins, skin, and muscles of the fish, and become metacercariae. The metacercariae are infective to humans and other fish-eating mammals upon ingestion of raw or undercooked fish in dishes such as "goi ca", and in turn the parasite's life cycle is completed. PCR techniques have been developed in recent years to detect small fluke eggs found in faeces and larvae of fish and snails. Detection of parasitic DNA by PCR and sequencing, realtime PCR is being considered to replace traditional diagnostic techniques [7]. Molecular biology techniques are more sensitive and specific than direct examination and immunological techniques even when the intensity of infection is low [11. However, these techniques are difficult to apply in health facilities because of the need to invest in modern laboratory systems, human resources training and expensive costs. Thus, molecular biology is still mainly used in laboratories [11][17][20].

The environmental DNA method (abbreviation: eDNA method) is a relatively new approach used to monitor the distribution of species. Using this method it is possible to detect species without actually seeing or catching them. The method uses DNA-based identification, also called barcoding, to detect species from extracellular DNA, or cell debris, that species leave behind in the environment. Research has shown that in water eDNA breaks down within a few days to a month. Therefore the detection of a species' DNA in the water confirms its recent presence. This methode is used for ecological surveys of different types of organisms and had been reported to be useful ecological tool for detecting target DNA in the environment, reducting time and costs. There are several study reported the detection of the eDNA of pathogenic organisms such as *Toxoplasma goldii*, *Cryptosporidium spp*, *Tereacapsuloides brysalmonae*, *Acanthamoeba*… and some of the study has examined the eDNA of Human trematode pathogen such as *Schitosoma japonicum*, *Opistochis viverrini* from water samples [[]]. In this study we applied a real-time PCR detection assay with specific species primers and a TaqMan probe targeting the COI and ITS1 regions of *O. viverrini* and *C. sinensis* to identify them from water and sedimentation feces samples.

2. SUBJECTS AND STUDY METHODS

2.1. Subjects: Faecal sediment collected in human positive with fluke by stool examinations and in water environment at the study sites.

2.2. Location, and duration of the study

2.2.1. Location of the study: The study was conducted in Chau Minh, Mai Trung and Hoang Vien communes, Hiep Hoa district, Bac Giang province and in My Tho, My Thanh and My Chanh communes, Phu My district, Binh Dinh province.

2.2.2. Duration: The study was conducted from January 2016 to December 2017.

2.3. Study methods

2.3.1. Study design: Descriptive cross sectional study

2.3.2. Sample size

Fifty sedimentation feces were positive for small-fluke egg were selected at the health stations was keep in 70° ethanol with rate 1 sediment: 1 ethanol and tranfer to Molecular biology Derpt of National Institute of Malariology, Parasitology and Entomology for real-time PCR analysis.

Fish culture water: A recent study by Japanese researcher Hashizume et al. (2017) developed a new method of condensing water samples in the bodies of water suspected for small fluke infection and highly sensitive primer pairs for detection of ADN in environmental samples [23]. Within the scope of the study, we intentionally chose water samples in fish ponds/lakes and the sample size in each commune was at least 20. Water samples were take from four different types of water bodies: ponds, lagoons, rice fields, canals. Each bodies of water needs 5 water samples. Thus, in all six communes we collected a total of 120 water samples for molecular biology techniques. Each sampling point two water samples were collected in 500 ml stereile bottles. After sampling the samples were added 1ml 1% Belzakonium chloric and tranffered to laboratory of National Institute of Malariology, Parasitology and Entomology for real-time PCR analysis

2.4. Techniques used in the study

For the isolation *O. viverrini* and *C. sinensis* in water and sediment feces, samples of water and sediment were filtrated through memberane filter (diameter 50 mm) with a pore 1,2 µm by means of a vacuum device. For the recovery of particles, the filter was rinsed by 10 ml of 0,1% PBS-tween 80, and centrifugation at 3000g for 10 minutes the sedimented pelled about 0,5-1 ml was subject for DNA extraction.

DNA extraction from faecal samples, and water sediment samples using the QIAamp DNA Mini Kit according to the manufacturer's instructions, based on the principle of using silica columns containing a silica resin that selectively binds DNA.

We use two separate real-time PCR methods. The first was Taq man Real-time PCR technique to identify *O. viverrini* and *C. sinensis*: using the pairs of primers targeting COI gene to identify *O . viverrini* [23]. OV-COI-F: 5' GCTGG ATTTG GGCAC CG-3' và OV-COI-R: 5' AGTAC CCGCA AGCAT ATACA ACC-3'; Probe: 5' FAM- TAGCT CGGTT ACTAT GATTAT –NFQ-MGB-3'. The primer sequence to identify *C. sinensis* on the target gene ITS1 designed by Cai X. Q. et al., 2012 [5] using the pairs of primers CS-ITS-F: 5' AGCGA TTCTA GTTCC GTCAT CTG-3'and CS-ITS-R 5' AGGTA TAGCC AGGAA TTGGT AGAAC-3' with Probe 5' FAM- CGCTC CACCG TAGGC ACAC –CSFQ-3'. And the second was Real-time PCR with high resolution melting analysis according to cai X. Q et al (2014): using the pairs of primers targeting COXI gene to identify *O . viverrini* and *C. sinensis*: COX1e-F: 5'-GGTAGGGTGGTTTGAGC-3'and COX1e-F: 5' TCATAGTAACC GAGCTAAA-3'. Melting peaks of *C. sinensis Tm* are: 79.05 ± 0.07°C; *O . viverrini Tm* are: 80.89 ± 0.06°C

3. RESULTS

3.1. Results of species minute fluke identification in human flukes by real –time PCR

3.1.1 Minute fluke species rate in humans by Real-time PCR

The results in table 1 showed that the mixed injection rates were higher than single infection rate, follow by 68.0% and 32.0%. In Bac Giang, the single infection of intestinal small fluke rates were 26.9% *H. taichui*, 3.8% *C. seninsis* and 3.8% *H. pumilio*. Mixed injection rates were with 30.8% of between liver fluke (*C. seninsis)* and minute intestinal flukes (*H. taichui* and *H. pumilio)* and 34.6% of both kind of minute intestinal flukes (*H. taichui* and *H. pumilio)*. In Binh Dinh, the single infection of intestinal small fluke rates were highest by 12.5% *H. taichui*, followed by 8.3% *O. viverrini* and 4.2% *H. pumilio*. Mixed injection rates were with 45.8% of both kind of minute intestinal flukes (*H. taichui* and *H. pumilio),* 16.6% of of between liver fluke (*O. viverrini)* and minute intestinal flukes (*H. taichui* and *H. pumilio)* and 8.3% of of between liver fluke (*C. seninsis)* and minute intestinal flukes (*H. taichui* and *H. pumilio)*.

Results study in table 2 showed that the overal prevalence of *H. taichui*, *H. pumillio* and *C. sinensis*, in Bac Giang by real-time PCR and PCR analysis of 26 positive stool samples was common 92,3%, 69,2% và 34,6%, respectively. In Binh Dinh, there were two species of small liver flukes, namely *C. sinensis* and *O. viverrini* with the prevalence of 8.3% and 29.2%, respectively, and the prevalence of *H. taichui* and *H. pumillio* was 83.3 and 75.0% respectively.

There were nine adult flukes samples collected on humans including four in Bac Giang and five in Binh Dinh. In Bac Giang, four small fluke samples were collected, in which two small liver flukes were *C. sinensis*, and two small intestinal flukes were identified as one infection of *Haploichis taichui* and one other mixed infection of *H. taichui* and *H. pumilio*. In Binh Dinh, five samples were collected including three small liver flukes (02 of *C. sinensis* and 01 of *O. viverini*) and two small intestinal flukes (both identified as *Haploichis taichui*).

3.1.2 DNA sequencing results from specimens

The analyzed sequence was 99.9% similar to that of *O. viverrini* and 100.0% similar to that of *C. sinensis* in genbank. Sequencing results were completely matched with real-time PCR and PCR analysis (Figure 4-5).

3.2. PCR results of water samples infected with small fluke larvae

An analysis of 120 water samples collected in Bac Giang and Binh Dinh was presented in the following table 3. In both the provinces, the analysis showed the presence of small fluke ADN in water samples at the study sites. The overall prevalence of minute intestinal flukein water was 20.0% (24/120). The prevalence in Bac Giang and Binh Dinh was 16.7% and 23.3% respectively.

4. DISSCUSION

Real-time PCR were used to identify MF species in humans aecal sediment positive for MFs, and pond/aquarium water samples. The results showed that the mixed injection rates were higher than single infection rate, follow by 68.0% and 32.0%. There have been found three species of human minute intestinal Bac Giang including H. taichui, *C. sinensis*, and H. pumillio with the respective prevalence of 26.7%, 3.8%, and 3.8%. Mixed injection rates were with 30.8% of combine C. seninsis, *H. taichui* and *H. pumilio* and 34.6% of combine *H. taichui* and *H. pumilio*. While at Binh Dinh, there have been found four species of human intestinal small fluke were with rate of 12.5% H. taichui, 8.3% O. viverrini and 4.2% *H. pumilio*. Mixed injection rates were with 45.8% of *H. taichui* and *H. pumilio*, 16.6% of combine O. viverrini, *H. taichui* and *H. pumilio*, and 8.3% of combine C. seninsis, *H. taichui* and *H. pumilio*. This result is similar to that of Woon-MokSohn found in the surveyed population of Luang Prabang province, Laos in 2014, the positive rate for small trematode eggs, which may include *H. taichui* and other heterophyids, Opisthorchis viverrini, and lecithodendriids, was 15.2%. Small fluke egg were verified to be those of intestinal fukes, particularly *H. taichui* [14]. The hyperendemicity of *H. taichui* infection among people in Bac Giang (92.3%) and Binh Dinh 87.2%) was closely correlated with the report of metacercarial prevalence in fish in both provinces with Bac Giang and Binh Dinh was 16.7% and 23.3%, respectively.

When analyzing water samples, results showed the presence of small fluke ADN in water sources at the study sites with low prevalences of 16.7% and 23.3%. In Hiep Hoa, Bac Giang, the water samples containing ADN of minute intestinal fluke mainly came from ponds around houses. In contrast, in Binh Dinh, water from large lakes and lagoons was identified as having small fluke infections. Therefore, in this study we sought to apply a novel species-specific real-time PCR assay for detecting O. viverrini using environmental DNA by Japanese researcher Hashizume et al. (2017) [11]. The diagnostic sensitivity of the newly developed real-time PCR assay was similar to that of the traditional PCR assay for 50 fecal

samples collected in Lao PDR with 21 and 19 samples were positive by real-time PCR and traditional PCR, respectively. The efficacy of eDNA analysis and its applicability in the field were tested using a total of 94 environmental water samples collected from 44 sites in Savannakhet, Lao PDR during May and October 2015 and February 2016. *O. viverrini* eDNA was detected in five samples by real-time PCR, indicating the presence of the fluke in the area and the risk of infection for individuals consuming fish from these water sources [11]. Results of gene sequencing showed that there was a similarity in nucleotide sequences among individuals of the same species in different study sites. There were no differences in nucleotide sequences at study sites and between individuals. The species of *O. viverrini* in Binh Dinh had the similar nucleotide sequence of 99.8% - 100% with samples on genbank. Small fluke species in Bac Giang included *C. sinensis*, *H. taichui*, and *H. pumilio*, with 100% homologous nucleotide sequence with the samples on genbank. This result is similar to that by Le Thanh Hoa (2002), which identified two main species of small liver flukes of the family Opisthorchiidae in Vietnam, *C. sinensis* in the North and *O. viverrini* in the South [14]. In addition, our study results are also consistent with the results by Ngo Van Thanh (2016) which investigated the prevalence of small fluke infection in Thanh Hoa and found the species of *C. sinensis* [5]. Its nucleotide sequence was 100% homologous with *C. sinensis* samples collected in Nam Dinh and stored in genbank. The human small intestinal flukes in Thanh Hoa were identified as *H. taichui* and *H. pumilio*, of which the nucleotide sequence was 99.8% - 100% similar to that of munite intestinal flukes collected in Nam Dinh and Thai Nguyen and stored in genbank **[19]**. Additionally, according to Do Trung Dung (2014), some species of small intestinal flukes were identified using molecular biology technique including *H. taichui*, *H. pumilio*, *Stellantchasmus falcatus*, *Centrocestus formoscanus*, and some other species of minute intestinal fluke such as *C. sinensis*, *O. viverrini*, *Echinochasmus japonicus* in 9 provinces of Vietnam **[8]**. In Binh Dinh, there were 4 species of *C. sinensis*, *O. viverrini*, *H. taichui* and *H. pumillio*, in which the prevalence of *C. sinensis* and *O. viverrini* was 3.0% and 21.3% respectively. Prior to this study, there were no reports of *C. sinensis* in Binh Dinh. With this result, it is the first time *C. sinensis* has been detected in the Central Highlands, and the South of Vietnam because researchers previously confirmed that small liver flukes in Vietnam were regionally distributed, i.e. *C. sinensis* mainly in the North, *O. viverrini* mainly in the Central Highlands and the South Vietnam [22]. This may be explained by the phenomenon of population movement between the North and the South. The intermediate hosts of small liver flukes are fish which are available in both the regions and at some time two species of small liver flukes will be distributed together. This promotes continued research to determine the distribution of human small liver flukes in different regions to help effectively diagnose and treat small liver fluke infections.

5. CONCLUSION

The study results confirmed by molecular biology revealed that:

- The results showed that the mixed injection rates were higher than single infection rate, follow by 68.0% and 32.0%.
- The infection load for trematodes was exclusively highest for *H. taichui*, follow by *H. pumillio*, *C. sinensis*, and *O. viverrini*.
- The overall rate of ADN minute intestinal fluke in water was 20.0%
- In this study, freshwater fish were infected with the minute intestinal fluke larvae at high rates with 12.5% to 95.0%.

RECOMMENDATION

It is necessary to continue to conduct community research to apply molecular biology techniques to determine the composition of human minute intestinal fluke pathogens in Vietnam. There should be further research on the prevalence of small fluke larval infection in fish ponds/lakes, the shift between small fluke species before climate change effects and current widespread integration and exchanges to obtain an accurate and reliable epidemiological map of small fluke distribution.

Table 1. Rate of single infection and mixed infections of the fluke by species in sediment

Kinds of infection No.(%)	Bac Giang prov.	Binh Dinh prov.	Total
No of samples (N)	26	24	50
Single infection	9 (34.6)	7 (29.2)	16 (32.0)
- *C. seninsis (Cs)*	1(3.8)		1 (2.0)
- *O. viverrini (Ov)*		2 (8.3)	2 (4.0)
- *H. taichui (Ht)*	7 (26.9)	4 (12.5)	11 (22.0)
- *H. pumilio (Hp)*	1 (3.8)	1 (4.2)	2 (4.0)
Mixed infection:	17 (65.4)	17 (70.8)	34 (68.0)
- Cs + Ht + Hp	8 (30.8)	2 (8.3)	10 (20.0)
- Ht + Hp	9 (34.6)	11 (45.8)	20 (40.0)
- Ov + Ht + Hp		4 (16.6)	4 (8.0)
- Ov + Ht		1 (4.2)	1 (2.0)

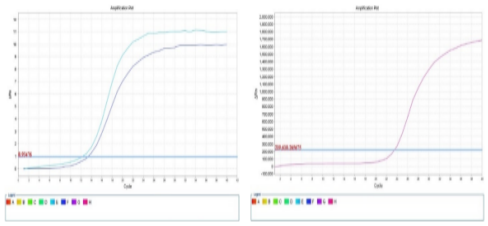
Table 2. The prevalence of each human minute intestinal fluke infection

Study site (Prov.)	No. of samples	Mfs infection - No.(%)			
		C. sinensis	*O. viverrini*	*H. taichui*	*H. pumillio*
Bac Giang	26	9 (34.6)	0 (0.0)	24 (92.3)	18 (69.2)
Binh Dinh	24	2 (8.3)	7 (29.2)	20 (83.3)	18 (75.0)
Total	50	11 (22.0)	7 (14.0)	44 (88.0)	36 (72.0)

Table 3. PCR analysis of water samples at the study sites

Locality	No. of samples	Small fluke species					
		C. sinensis	*O. viverrini*	*H. taichui*	*H. pumillio*	No. of pos.	Per. (%)
Bac Giang	60	2	0	6	2	10	16,7
Binh Dinh	60	0	4	5	5	14	23,3
Total	120	2	4	11	7	24	20,0

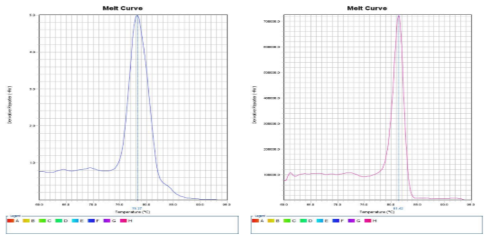
Figure 2. Results of the identification of small liver flukes by Taq man real-time PCR



A B

Positive for *C. sinensis* in Bac Giang and Binh Dinh; B. Positive for *O. viverrini*

Figure 3. Identification of small liver flukes by real-time PCR using melting curve analysis



A B

Positive for *C. sinensis;* B. Positive for *O. viverrini*

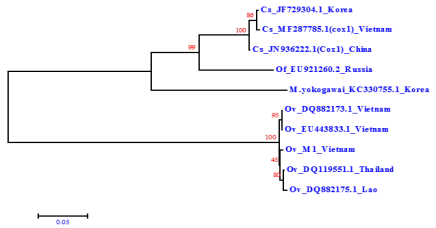


Figure 4. Generalogical relationship between *O. viverrini* with international strains based on obtained sequences

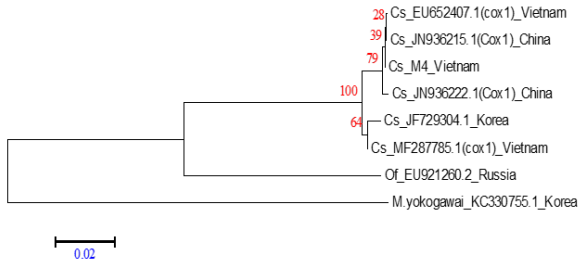


Figure 5. Generalogical relationship between *C. sinensis* with international strains based on obtained sequences

REFERENCES

1. Aung W. P. P., Thi T. H., Kyi k. T., et al (2017). First report and molecular identification of Opisthorchis viverrini infection in human communities from lower Myanmar. PLOS One 12(5): e 0177130.
2. Bass D., Grant D. S., et al (2015). Diverse applications of Enviromental DNA methods in parasitology. Trands in Parasitology: Vol 31 (10:)499-513
3. Boerlage AS. (2013), Transmission and control of Fish-borne Zoonotic Trematodes in aquaculture, PhD thesis, Wageningen, 150p.
4. Bui Ngoc Thanh (2017), The research actuality of the larva expels leaves are capable of transmission to people infected on the fish in the mountains of northern Vietnam, Doctoral thesis in Biology, Academy of science and technology, National Academy of science and technology of Vietnam, 134 pages.
5. Cai X. Q., Hai Q. Y., Rong L., et al (2014). Rapid detection and differentiation of Clonorchis sinensis and Opisthorchis viverrini using real-time PCR and high resoludtion Melting analysis. The Scientific World Journal, Volume 2014, Article ID 893981, 6 pages http://dx.doi.org/10.1155/2014/893981
6. Chai JY, Murrell KD, Lymbry A. Fishborne parasitic zoonoses: Status and issues. Int J Parasitol. 2005;35:1233–1254.
7. Denier K., Holly M. B., Elvira M., et al. (2017). Enviromental DNA metabarcoding: Transforming how we wurvey animal and plant communities. Molecular ecology: 5872-5895.
8. Do Trung dung (2014), The study of the molecular and morphological characteristics of leaves of the small intestine t on people in some provinces and effectively treated in the community, in 2010-2013, Ph.D. thesis, National Institute of Malariology-Parasitology and Entomology, 162 p.
9. Doanh P.N; Yukifumi Nawa (2016). Clonorchis sinensis and Opistochis spp. In Vietnam: current status and prospects. Trans R Soc Trop Med Hyg: 110: 13-20.
10. Dorny P., Praet N., Deckers N., Gabriel S., (2009) "Emerging food-borne parasites", Vet Parasitol, 163(3), pp.196-206. doi: 10.1016/j.vetpar.2009.05.026. Epub 2009 Jun 6
11. Hashizume H., Sato M., Sato M. et al (2017), "Application of environmental DNA analysis for the detection ofOpisthorchis viverrini DNA in water samples", Acta tropica, 169, pp. 1-7.
12. Hung N. M., Do T. D., Nguyen T. L. A., at al (2015). Current status of fish –borne zoonotic tramatode infection in Gia Vien district, Ninh Binh Province, Viet nam. Parasitology & Vector 8: 21.
13. Jeon HK., Lee D., Park H. et al (2012), "Human Infections with Liver and Minute Intestinal Flukes in Guangxi, China: Analysis by DNA Sequencing, Ultrasonography, and Immunoaffinity Chromatography", The Korean Journal of Parasitology. 50(4), tr. 391-394.
14. Le Thanh Hoa, Nguyen Van De, Nguyen Van Bich, Russia (2002), "Judge expels Clonorchis sinensis baby liver species in Vietnam by molecular genetics company", Journal of malaria and parasite diseases control. 4, tr. 60 - 68.
15. Liu D. (2012), Molecular Detection of Human Parasitic Pathogens, CRC Press, 895p.
16. Mahmoudi M. R., Bahram K., Ali H., et al (2015). Detection of Acanthamoeba and Toxoplasma in river water samples by molecular methods in Iran. Iran J Parasitol: Vol 10, No. 2: 250-257.
17. Mahmoudi M. R., Mojgan B., Bahram K., et al (2017). Detection and enumeration of cryptosporidium oocysts in environmental water samples by real-time PCR assay. J Bas Res Med Sci 2017: 4(3): 42-47.
18. Men-Bao Q., Ying-Dan C., Yue-Yi F.I (2013), "Epidemiological profile ofClonorchis sinensis infection in one community, Guangdong, People's Republic of China", Parasites & Vectors, 6:194.
19. Ngo Van Thanh, Vu Hong Cuong and Nguyen Van De (2014), "Status on infections through freshwater fish parasite on people in four communes coastal in Nga Son district, Thanh Hoa province, in 2013". The proceeding report on the 20th conference PhD student Science Research in Hanoi. Hanoi Medical University, pp 138-139.
20. Nguyen Thu Huong, Tran Thanh Duong (2013), "The study describes the case of small liver Fasciola gigantica disease lost a rare spot by method of molecular biology in Hanoi, in 2013", Journal of Practice medicine, 886, pp. 57 – 62.
21. Phan Thi Van, Bui Ngoc Thanh (2013), Fishborn Treamatoda in Vietnam, agricultural Publishing House, 87 pages.
22. The Ministry of health (2015), Parasite epidemiology in medicine, Medical publishers, Hanoi, pp. 424-427.
23. Traub R. J., Julie M., Mathirut M., et al. (2009). A new PCR-based approach indicates the range of Clonorchis sinensis now extends to Cntral Thailand. PLOS neglected tropical diseases. V3. (1)e 367.
24. Woon-MokSohn, Tai-SoonYong, Keeseon S.Eom, Duk-Young Min,Dongmin Lee,Bong-Kwang Jung, Virasack Banouvong, Bounnaloth Insisiengmay, Bounlay Phommasack, Han-JongRim, Jong-YilCha (2014), "Prevalence of Haplorchis taichui among humans and fish in Luang Prabang Province, Lao PDR", Acta Tropica, 136, pp. 74-80.