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Molecular Method vs. Traditional Methods for Estimating the Prevalence of Larval Trematode Infections in Some Red Sea Snails

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ABSTRACT

The importance of marine snails in the transfer of cercariae to fish as a provenance of zoonoses should be considered. Parasitic studies in marine snails have been uncommon in Saudi Arabia. In the present study, 550 Nerita genus of marine snails were monthly and randomly collected from January to December 2016 from Obhor bay, Saudi Arabia. The snails were subjected to light. They were crushed to evaluate the presence of larval trematodes. PCR technique was performed using the internal transcribed spacer region of ribosomal DNA (ITS-rDNA) which has been a specific primer to detect the extent of larval trematode infection in some snails infected as positive control samples and all non-infected snails to assess if they were infected or not. PCR technique showed a high prevalence of infection (55.82%) than the classical methods (21.45%). These studies can help in collecting data on the ecological importance of the distribution of disease in sympathetic fish, and the transmission of digenean trematodes disease through snails. Moreover, they can be useful in preventing and control of fish and human diseases.

Key words: *Marine snails, Prevalence, Trematode larvae, Cercaria, Molecular.*

INTRODUCTION

Snails belong to gastropod, which represent a large and highly diverse group of mollusca. They serve as intermediate hosts for various trematode parasites, where several developing larval stages are developed such as sporocysts, rediae, and cercariae.

Percentage of snails that liberate cercariae (infection) and the number of releases from each infected snail play important roles in the transfer of trematodes from the snail host [1, 2]. Trematodes are a set of parasites which need intermediate and definitive hosts such as molluscs and vertebrates.

The parasitic trematode is often related to changes in host growth, fecundity, survival, and snail susceptibility [3]. Usually, snails are separated into small containers, incubated under constant light and temperature, and cercariae are identified under stereomicroscope and its prevalence is recorded [4, 5]. When alive snails are not needed, crushing method may be used [6, 7].

Not surprisingly, cercarial shedding as means of detection was criticized as an inaccurate method by different previous studies [8, 9]. In a comparison between the estimation of prevalence obtained from both cercarial shedding and snail crushing, the prevalence rate increased with the crushing method, including also the multiple infections detection rates [10, 11].

Furthermore, mature cercariae did not shed in some snails [12]. The double infection is more difficult to be detected because synchronous production of two different species types of cercariae in the same snail is less successful in individual infections, possibly due to competition for host resources [13-15]. Caron [16] indicated the importance of PCR-based techniques used in investigating the prevalence of infection in snail hosts. A

double PCR assay for two host-parasite systems enables them to distinguish specifically between individual and double infections. This method could also specifically amplify parts of different sizes of the ITS rDNA for various species of larval trematode infecting the digestive glands or gonads of the same snail [17- 20].

It is important to point out that, until now there is no available single technique, which is simple, robust, reproducible and cheap enough to detect accurate infection. PCR also detects parasite invasion sufficiently. The microscopic examination has achieved successful infections in the snail host. Both techniques can be used together to achieve a more comprehensive understanding of the epidemiological situation in a given area and to assess the ability of different intermediate hosts to maintain larval development [21]. They enable highly sensitive PCR assay, early invention of infection and the feasibility of large-scale assay of snails with minimal effort [22, 23]. Few studies have been conducted abroad on the variety and amplitude of infection by cercaria in Red Sea snails. Therefore, the current study aimed to elucidate the prevalence of cercarial infections of some Nerita genus of marine snails in Jeddah coast using classical methods, detect the infection prevalence in the same snails using molecular method and then compare between the prevalence of infection prevalence obtained by the classical and molecular methods.

MATERIALS AND METHODS:

Collection of snails: *Nerita* snails were gathered from Obhur bay, 30 km from the east coast of the Red Sea north of Jeddah city and were transferred to the Parasitology laboratory, Biology Department, Science College, King Abdulaziz University, Jeddah during the interval from January to December 2016. The cercariae were shed and harvested.

The various species of snail were separated into samples in plastic containers, cleaned, placed in a petri dish which contain seawater, kept at 23°C, and then naked for two hours to artificial light. Finally, the water in each dish was examined at short periods using a binocular dissecting microscope for the appearance of the cercariae.

To facilitate the appearance of cercariae, in 250 ml beaker the infected snails were separated and placed, the non-shedding snails were crushed and examined to look for early larval trematode stages. All the collected samples of *Nerita* snails whether infected or non-infected with larval trematode were subjected to DNA extraction using DNeasy Blood and Tissue Kit (Qiagen) [25].

PCR protocol: Preparing 25µl reaction for PCR by mixing: 1 µl DNA of each sample in separate PCR tube + Master mix 12.5 µl + 1 µl of Forward primer + 1 µl of Reverse primer + Nuclease-free water up to 25 µl. DNA was amplified by PCR according to [24]. It was done using the primers ITS5 forward (5- GGA AGT AAA AGT CG AAC AAG-3) and ITS4 reverse (5-TCC TCC GCT TAG TGA TAT GC-3) according to [26].

All samples were placed in a PCR machine. The program conditions for PCR were 94° C for 2 minutes followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 50°C, and 1 minute at 72°C. After 35 cycles, the temperature was set to 72 °C for 10 minutes. The PCR products were analyzed on a 2% agarose gel and stained with 5µl Ethidium Bromide (EtBr).

Bands on the gel were scanned as digitalized images. PCR products were visualized in 5 µl aliquots after running on a 1% agarose gel.

Chi-square was used to analyze and compare the rate of infection and differences in snails' species between the infected and non-infected snails. They were tested each month and for length. SPSS software (version 22) was performed for statistical analysis. Probability of $P < 0.05$ was considered significant.

RESULTS

Figures 1, 2, 3, 4, 5, 6, 7 and 8 show the prevalence of Infection in Snail Hosts using Shedding and Crushing Methods: A total of 550 *Nerita* snails were randomly selected from Obhor bay at Jeddah coast. The snails were classified based on the morphology of seashells using modern keys and were found to belong to Neritidae and genus Nerita family.

From the genus Nerita, six species were identified and examined (189 of *Nerita albicilla*, 91 of N. *grayana*, 53 of *N. polita*, 93 of N. *quadricolor*, 52 of N. *orbignyana* and 72 of N. *histrio*). As shown in Table (1), the highest trematode infection rate for all *Nerita* snails was observed in May with percentage of 64% but no infections were found in March.

Table 1: The prevalence of infections detected by the classical methods compared to the molecular method

Months	Total no. snails	No. infected by classical methods (%)	No. infection by molecular methods	P value
January	70	5 7.14%	63 90%	0.00*
February	70	3 4.29%	54 77.14%	0.00*
March	25	0	15 60%	0.00*
April	30	4 13.33%	16 53.33%	0.01*
May	25	16 64%	20 80%	0.173
June	45	18 40%	32 71.11%	0.03*
July	34	2 5.88%	10 29.41%	0.01*
August	34	1 2.94%	7 20.59%	0.02*
September	34	7 20.59%	10 29.41%	0.287
October	45	6 13.33%	9 20%	0.86
November	71	44 61.97%	49 69.01%	0.06
December	67	12 17.91%	51 76.11%	0.00*

The mean prevalence of larval trematode infections detected by shedding and crushing methods was 21.45%. Prevalence of infection in snail hosts using PCR method: For the used ITS5 forward and reverse primer sequences, nucleotide Basic Local Alignment Search Tool (BLAST) has been done using larval trematode as a target.

The used primer pair gives different product sizes ranged from 107- 1851 bp, with the same product size in some different trematode species. In the current study, some samples gave single, double, triple, quadruple, quintuple and hexagonal bands after doing PCR using the mentioned primer pair, which meant that each band might represent at least only one infection, with different snails having various number of bands, which indicate multiple infections in the same snail (Figures 1, 2, 3,4,5 6,7 and 8).

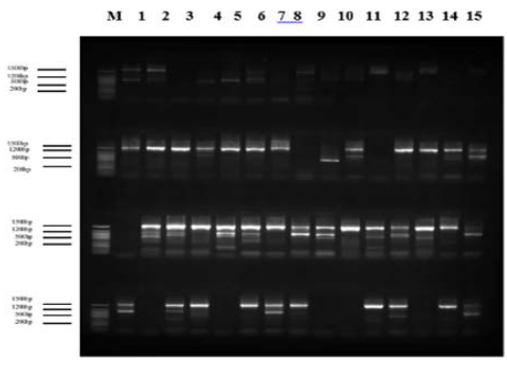

Figure 1: Molecular results for infection prevalence: Agarose gel for PCR detection of larval trematode infection in 60 out of 70 *Nerita* snails collected during January 2016 based on ITS5 specific primer pair. The first five lanes of the first row represent infected snails (positive control). M refers to 50bp ladder.

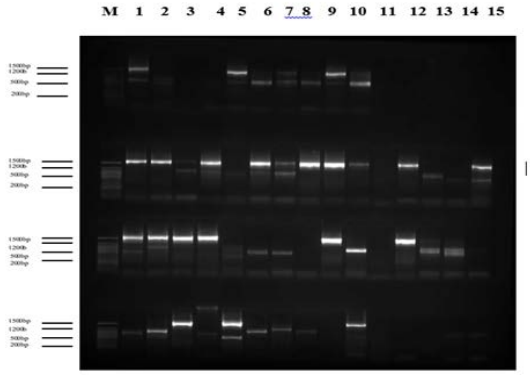

Figure 2: Molecular results for infection prevalence: Agarose gel for PCR detection of larval trematode infection in 10 out of 70 *Nerita* snails collected during January (first row) and 45 out 70 collected during February (second, third and fourth rows) 2016 based on ITS5 specific primer pair. The first three lanes of the second row represent infected snails (positive control)

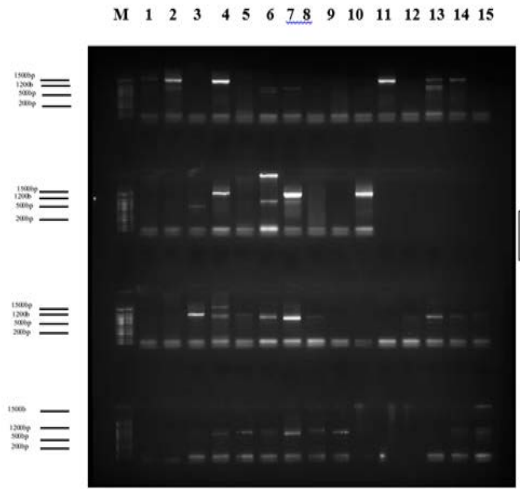

Figure 3: Molecular results for infection prevalence: Agarose gel for PCR detection of larval trematode infection in the rest 25 Nerita snails collected during February (First and second row), 25 out 25 collected during March and 5 out 26 collected during April (fourth rows) based on ITS5 specific primer pair. M refers to 50bp ladder.

for February. M refers to 50bp ladder

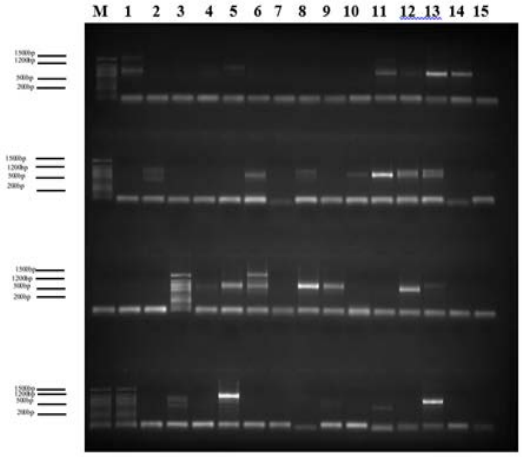

Figure 4: Molecular results for infection prevalence: Agarose gel for PCR detection of larval trematode infection in 21 out of 26 *Nerita* snails collected during April (the whole of first row and five lanes of second rows), nine out of 12 collected during May (second row), the third row represents three out 12 collected snails during May, 50bp ladder and then three infected snails as positive control collected during June and the rest of the third row and fourth row are the rest of June samples, based on ITS5 specific primer pair. M refers to 50bp ladder

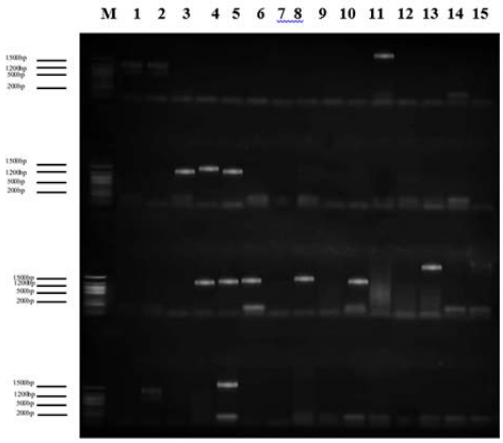

Figure 5: Molecular results for infection prevalence: Agarose gel for PCR detection of larval trematode infection *Nerita* snails, the first four lanes of the first row are the rest of June samples, two infected snails as positive control collected during July and the rest of the first row lanes, the whole second row lanes in addition to the first eight lanes of the third row. Lane number 9 of the third row represents infected snail collected during August and the rest of the third row lanes and the complete fourth row lanes represents the rest of August snail samples, based on ITS5 specific primer pair. M refers to 50bp ladder.

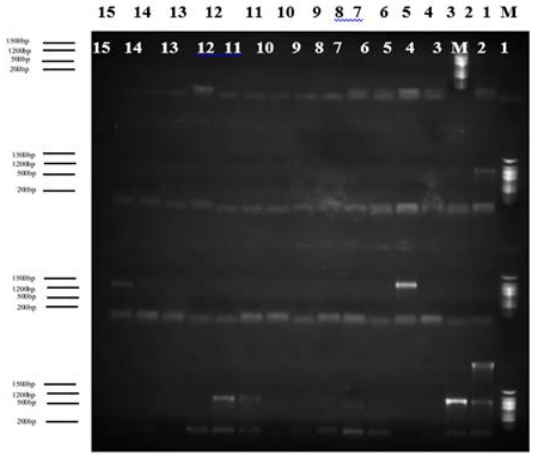

Figure 6: Molecular results for infection prevalence: Agarose gel for PCR detection for larval trematode infection in 12 of rest of sample for August in Nerita snails collected during August (first row), 50bp ladder and then three infected snails as positive control collected during September 2016. In second row five sample infected snail during in September the rest row two, three and two lanes of four row non- infection, 50bp ladder and five lanes of infected during October, rest raw the non-infected snails using classical method. M refers to 50bp ladder.

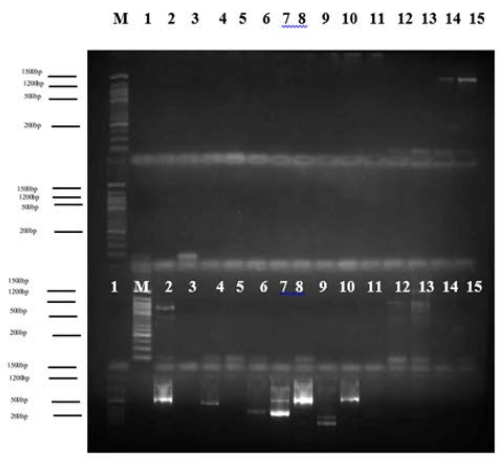

Figure 7: Molecular results for infection prevalence: Agarose gel for PCR detection of larval trematode infection in *Nerita* snails collected during October the whole of first row and second row and first leans in row three are non- infected, 50bp ladder and then two infected snails as positive control collected during November and the rest of the third row and fourth row are the rest of November samples non-infected. M refers to 50bp ladder.

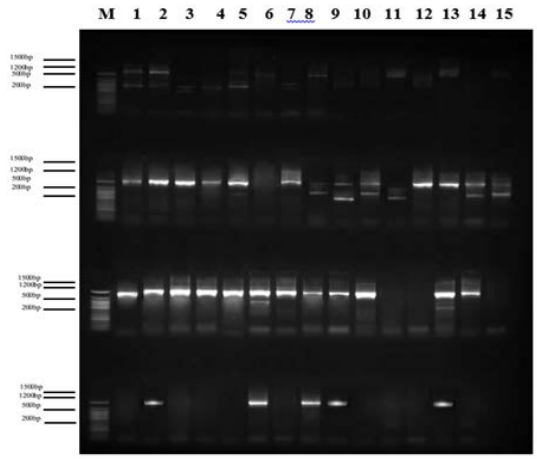

Figure 8: Molecular results for infection prevalence: Agarose gel for PCR detection of larval trematode infection in *Nerita* snails two leans during November. Five lanes of first rows infected snails as positive control collected during December. The second, third and four row represents collected snails during November. M refers to 50bp ladder.

In the molecular level, some of the infected snails were used as positive control in addition to all non-infected snails to make sure about their infection. As shown in Table (1), the highest trematode infection rate for all Nerita snails was observed in January with percentage of 90% but the lowest infection rate was found in October with percentage of 20%. The mean prevalence of larval trematode infections detected by shedding and crushing methods was 55.82%.

In the current study, the applied classical and molecular methods were compared using Chi-square test. As shown in Table (1), the results of Chi-square test showed statistically significances between the prevalence. All the comparisons between both methods showed that the molecular method using PCR was significantly higher in prevalence than the classical methods in January, February, March, April, June, July, August, and December, but non-significant differences were found in May, September, October and November.

DISCUSSION

The most significant factor which affects the appearance of infection by larval trematode in marine water is the accessibility of appropriate snail host. Snails act as intermediate hosts and play a significant part in the transmission of trematode species so that, the trematode's life cycle (Sprocysts, rediae and cercariae) is completed inside it.

In the Red Sea, several marine snail species were found. They serve as intermediate hosts for various trematodes that affect the stock of our lives and birds. In the current study, the overall prevalence of larval trematodes in all Nerita species using shedding and crushing methods was 21.45%. In classical studies, evaluating the prevalence of snails' infection using the crushing method may provide more dependable results than those depended on shedding of cercaria following incubation [10].

However, the present study revealed that, the crushing method might not be accurate enough, especially to detect juvenile, double, and multiple infections. To the best of our knowledge, this is the first study in which the ITS5 specific primers have been designed in a PCR to accurate assessment of single, double and multiple infections depending on the produced product sizes. The PCR based prevalence results revealed 55.82% of larval trematode infections in all Nerita species compared to the 22% produced by crushing method. Our results are consistent with Caron [27] who highlighted the significance of PCR-based techniques that enable them to distinguish single and double infections. They also reported that, this method could also specifically amplify differentially sized segments of ITS rDNA for different larval trematode species which infect the same snail

host.

Inaccurate detection using crushing method is possibly due to immature infections and the high occurrence of non-identified multiple infections in specimens, which might arise from difficulties with the identification of the morphology of closely related trematode species. Due to increased accuracy of the PCR method, this could consequently change our understanding of the larval trematode community structure in snail hosts provided that a stronger valuation of the importance of competition between species within the snail host exists and to propose other mechanisms that might facilitate multiple infections.

The current study results are also in agreement with previous studies, which used primers specific to species for detection and identification of infections by larval [28, 29]. In addition, [30] documented that PCR-based method is more accurate compared to cercarial shedding method using specific primers for the detection of Dicrocoelium dendriticum single infections in snail tissues. Morover, [30] used a mtDNA multiplex PCR to discriminate Caliophoron daubneyi and Fasciola hepatica in the snail Galba truncatula; otherwise, they could not detect double infections, neither by microscopy nor by PCR, while in the current study we documented single, double and multiple infections by PCR only. Through this methodology, the low parasitic burden, immature or hidden infections and molluscs death do not prevent the estimation of the true infection prevalence. The magnitude of the impact of parasitism on snail hosts is often ignored or underestimated [6].

Minimizing the effects of parasitism can have complex consequences if the prevalence of infection or combinations of species is estimated to be multiple, for example in detecting potential seasonal variations [5]. Therefore, determining accurate prevalence of infection is exceptionally important.

Finally, the current study confirmed that the PCR method for detection of single and multiple infections demonstrates the superior accuracy of the PCR-based method than the traditional methods. It also avoids identifying erroneous data in the case of closely related species infecting snail host itself, or when there are immature larval stages. PCR methods could be efficient and fast methods for uncovering the actual prevalence of larval trematode infections in the snail hosts.

The use of molecular techniques to correctly detect the incidence of infection with different species of trematode is definitive not only because of its relationship with public health but also because of its significant assistance to the livestock production and national economy.

CONCLUSION AND RECOMMENDATIONS

The results of PCR technique showed higher infection prevalence (55.82%) compared to the classical methods (21.45%).

These studies can help to collect data on the ecological importance in the distribution of disease in sympatric fish and the transmission of digenean trematodes disease through snails as well as in preventing and control fish and human diseases.

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