Towards Understanding the Liver Fluke Transmission Dynamics on Farms: Detection of Liver Fluke Transmitting Snail and Liver Fluke-Specific Environmental DNA in Water Samples from an Irrigated Dairy Farm in Southeast Australia

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Abstract

Background

Livestock production around the world is impacted by liver fluke (*Fasciola* spp.) infection resulting in serious economic losses to the beef, dairy and sheep industries with significant losses of about $90 million per annum in Australia. Liver fluke infection is predominantly controlled by anthelmintic treatment and Triclabendazole (TCBZ) is usually the drug of choice due its superior efficacy against early immature, immature and adult liver fluke stages; however, the widespread emergence of TCBZ resistance in livestock threatens liver fluke control. We are in the urgent need for alternative control measures to lower the exposure of livestock to liver fluke infection which would help to preserve the usefulness of current anthelmintic treatments. Our ability to understand the prevalence of intermediate snail hosts and infective liver fluke stages in the environment is crucial to implement alternative control measures for liver fluke control. However, identification of liver fluke and snails in the environment is hampered by lack of efficient diagnostic methods. Environmental DNA (eDNA) based identification of liver fluke and the intermediate snail host in the water bodies is a promising method to identify liver fluke and snail prevalence on farms. Our aim is to provide a proof of concept to use a molecular tool (quantitative PCR) to detect and quantify eDNA of liver fluke and snail in water bodies on Victorian farming properties for potential large-scale analysis of liver fluke and snail ecology in water bodies.

Methods

To demonstrate the identification of liver fluke and snail in water bodies, we used a multiplex quantitative PCR assay for the independent but simultaneous detection of eDNA released from snail (*Austropeplea tomentosa)* a crucial intermediate snail host for liver fluke transmission in South-east Australia and free-living liver fluke stages (*Fasciola hepatica)*. We have collected water samples from an irrigation channel over a period of 11 months in 2016 at a dairy farm located at Maffra, Victoria, South-east Australia and used water samples from selected months (February, March, May, September, October, November and December) for eDNA assay.

Results

The multiplex qPCR assay effectively allows for the detection and quantification of eDNA released from liver fluke life stages and snails and we observed differential levels of liver fluke and snail specific eDNA in water at the time points analysed in this study. This assay was able to detect 14 fg and 50 pg of liver fluke and snail DNA in the presence of potential inhibitors from field collected water samples.

Conclusion

The successful detection of eDNA specific to liver fluke and snails from the field collected water samples provides a proof of concept for the use of this method as a monitoring tool to determine the prevalence of liver fluke and liver fluke-transmitting snails in irrigation regions to allow for understanding the liver fluke transmission zones on farms to implement effective control strategies.

1. Background

Fasciolosis or liver fluke disease is a food and waterborne parasitic disease caused by the liver flukes, *Fasciola hepatica* and *F. gigantica* [1, 2]. *Fasciola* spp. have a broad host range including a variety of livestock species, as well as humans. *Fasciola* infections cause significant production losses to the livestock industry worldwide: the recent reports of *Fasciola* infection in humans also make fasciolosis a public health issue [3, 4]. *Fasciola* spp. are estimated to infect over 600 million animals costing the livestock industry over US$3 billion p.a. in production losses [5]. In addition, *Fasciola* spp. are estimated to infect 17 million people worldwide and approximately 180 million people are at the risk of contracting *Fasciola* infection [3]. As a result of this, the WHO has classified fasciolosis as a 'neglected tropical disease' [6, 7]. *Fasciola* infection is primarily controlled by anthelmintic treatment and Triclabendazole (TCBZ) is considered as the most potent drug against liver flukes as it targets early immature and adult liver flukes [8]. However, *F. hepatica* has widespread TCBZ resistance worldwide and alternative control measures are needed to control TCBZ-resistant *F. hepatica* infections [9].

Fasciola spp. undergo a complex life cycle with aquatic snails as the intermediate host and several Lymnaeidae snail species have been reported as intermediate hosts for *Fasciola* transmission worldwide (reviewed in Correa et al., 2010) and the most commonly found intermediate host for *F. hepatica* in South-east Australia is *Austropeplea tomentosa* [10, 11]. The intermediate snail hosts grow and reproduce in water bodies, flood-irrigated pastures and wetlands; and therefore, creating a suitable environment for liver fluke transmission in these conditions. Estimation of intermediate snail host prevalence in water bodies/irrigation channels would provide valuable information to understand the dynamics of liver fluke transmission in an area and assist the development of integrated parasite management plan for liver fluke control. Currently, the intermediate snail hosts are identified by physical collection of snails in water bodies followed by speciation using microscopy or molecular analysis [12, 13, 14]. Identification of liver fluke transmitting snails on large-scale farms becomes notoriously difficult as the physical collection of snails is a time consuming and labour-intensive process. However, the difficulty in intermediate snail identification can be overcome using environmental DNA (eDNA) approach as eDNA based identification of organisms in water bodies has shown great promise to estimate biodiversity in natural and effluent waters [15, 16]. In the last decade, several macroorganisms have been identified in water bodies aiding in biodiversity estimation, detection of invasive species and monitoring of endangered species [15]. To implement such an approach to identify and monitor liver fluke transmitting snails and free-living liver fluke stages in water bodies on farms, we have recently developed a multiplex quantitative PCR assay to detect and quantify *A. tomentosa* and *F. hepatica* eDNA from water samples [17].

Our aim is to demonstrate the use of multiplex qPCR-based assay to detect and quantify eDNA released from snails and free-living liver fluke stages in water samples collected from a farming property. An eDNA-based snail and free living liver fluke stage identification program can be particularly useful to understand liver fluke prevalence in irrigated and rainfed areas as the risk of liver fluke infection is determined by the presence of the intermediate snail host [18]. To achieve this, we have selected a property in Maffra, Victoria, South-east Australia as the Maffra region has a very high prevalence of liver fluke infection with the mean prevalence of 81% [19]. Furthermore, future application of eDNA approach to determine the prevalence of the intermediate snail host (*A. tomentosa*) in these regions would potentially assist the control of liver fluke infections by allowing producers to segregate animals from snail infected water bodies.

Here, we demonstrate a proof of concept for using eDNA approach to identify and quantify eDNA released from snails and free-living liver fluke life stages in the water samples collected over an 11-month period from an irrigation channel on a dairy farm known to contain liver fluke infections at Maffra, Victoria, South-east Australia. This study is the first of its kind to quantify liver fluke and snail eDNA in water samples from a dairy farm and document the seasonal variation in the kinetics of eDNA appearance.

2. Methods

2.1 Study area

This research was conducted on a dairy farm with the herd size of 1000 dairy cattle in the Macalister Irrigation District (MID), Maffra, Victoria, Southeast Australia with the approval from LaTrobe University Animal Ethics Committee (AEC#14–51). The dairy farm selected in this study has been previously established to contain liver fluke infections (Kelley, unpublished). A single irrigation channel in the farm was selected for water sample collection. The irrigation channel (600 meter in length 1 meter in width) sampled in this study has been designated to irrigate 10.6 hectares (separated into 4 paddocks) and the whole channel was fenced on both sides with a tree line beside the channel. We sampled a part of the irrigation channel (150 meters) adjoining the paddock highlighted in the Fig. 1 and the water flows into this irrigation channel from Macalister Irrigation District supply channel. All the paddocks /irrigation channel have been laser graded to allow for effective water flow. Usually, the water flow has been still in the irrigation channel except during the irrigation. In addition, faecal samples from ten randomly selected cattle were collected on every water sample collection to confirm the presence of liver fluke infections on the farm.

2.2 Faecal egg counts

Faecal samples collected during water sample collection were processed to determine the liver fluke faecal egg counts (LFEC). The faecal samples were weighed (2 g) into a hexagonal sample bottle and filled with water. The samples were mixed and sieved using a 177 µm sieve into a 250 ml sedimentation flask and the faecal material was gently washed with water. The samples were allowed to sediment for 3

minutes and the water was poured until about 40 ml remained in the sedimentation flask. The sieving step was repeated and the water was poured until about 20 ml remained in the sedimentation flask. The sediment was washed into a 15 cm test tube and filled up with water followed by sedimentation for 3 minutes. The supernatant was carefully removed down to 2 cm, stained with 1–2 drops of 1% methylene blue followed by gentle agitation. The content of the test tube was poured into a perspex counting tray and examined under a stereo microscope at about 15x magnification.

2.3 Water sample collection

Water samples (~ 500 ml) were collected in a sterile single use plastic container from ten sites (approximately 10 meters apart) along the irrigation channel and inside the single water trough (WT). Sample collection containers has been prelabelled with sample collection site number, collection date and the personal involved, and sample collection containers were covered with parafilm after the sample collection. The water samples were collected every month from February 2016 to December 2016 to assess the snail and liver fluke eDNA levels in the irrigation channels. All the water samples were transported to LaTrobe University at 4 °C and stored at -20 °C until eDNA extraction. The available water samples were collected from the irrigation channel when the water movement was stopped for irrigation.

2.3 Snail collection and identification

Surface mud and debris were collected from each sampling site into a sieve and washed vigorously within the channel to remove any loose soil and snails were visually identified within the sediment mixture (10 minutes/sample collection site). Snails identified in the sediment mixture were collected and stored in irrigation water at -20 °C. Snails were defrosted at 4 °C prior to analysis and visually identified under a light microscope. Visual identification was based on the bulge and opening of the shell and the number of whorls present in the shell. From these features, snails were classified as either ‘liver fluke transmitting’ or ‘non-liver fluke transmitting’ snails. Snails were stored in plastic vials with 100% ethanol and labelled with collection date and site number for any further analysis.

2.4 DNA isolation

Adult liver fluke for DNA isolation were collected from the liver of experimentally infected rats [20]. A clean liver fluke was snap frozen in liquid nitrogen and homogenised into a fine powder using a mortar and pestle. Genomic DNA from the liver fluke was extracted using a DNeasy® Blood & Tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions and stored at -20 °C until further use.

Genomic DNA from snails (*A. tomentosa* obtained from Invetus, Armidale Research Centre, Armidale, NSW) was isolated according to Winnepenninckx et al (1993) with minor modifications. Briefly, snail tissue was collected from the shells and homogenised in lysis buffer (2% (w/v) Cethyl Trimethyl Ammonium Bromide (CTAB), 1.4 M NaCl, 0.2% (v/v) β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl pH 8 and 0.1 mg/ml proteinase K) and incubated at 60 °C for 2 hours with mixing every 15–30 minutes [21]. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to the suspension to precipitate the proteins and the suspension was centrifuged at 11,000 x *g* for 15 min at 4 °C. The

aqueous phase was carefully transferred to a new tube and an equal volume of chloroform: isoamylalcohol (24:1) was added, mixed with the sample and then centrifuged at 11,000 x *g* for 15 min at 4 °C. The DNA was precipitated by the addition of 2.5 volumes of cold 100% ethanol followed by an overnight incubation at -20 °C and centrifuged at 10,000 rpm for 15 min at 4 °C. The DNA pellet was washed with 70% ethanol and centrifuged at 10,000 rpm for 15 min at 4 °C. The pellet was air dried and resuspended in nuclease free water and stored at -20 °C. The concentration and purity of liver fluke and snail DNA were estimated using a Nanodrop™ 2000 spectrophotometer (Thermo Scientific, USA). Furthermore, the ITS-2 region of *A. lessoni*, *P. acuta* and *G. truncatula* were synthesised and cloned into the pBHA vector by Bioneer Pacific, Australia, due to the unavailability of the biological material from *Austropeplea lessoni*, *Physa acuta* and *G. truncatula* for DNA extraction.

2.5 Environmental DNA isolation

The irrigation water samples stored at -20 °C were defrosted at 4 °C for eDNA isolation. We performed environmental DNA extractions in a clean lab area without snail or liver fluke DNA presence and positive and negative controls have been used during eDNA isolation. The eDNA isolation were performed in Centre for AgriBioscience, LaTrobe University and we have used dedicated facilities for DNA isolation, PCR reaction set up and PCR analysis to avoid cross contamination. Ten ml aliquots were taken from each water sample and 0.1 µg of plasmid DNA encoding the internal transcribed spacer (ITS-2) region of *Galba truncatula* was added as an internal DNA extraction control to each aliquot of water sample. The eDNA purification method was adapted from Li and Sheen (2012) as described (Rathinasamy et al., 2018). Briefly, two volumes of binding solution (6M NaI, Sigma-Aldrich) and 100 µl of silica matrix (100 mg/ml SiO2, Sigma-Aldrich, USA) were added to each 10 ml water sample and mixed on a rocker for an hour at room temperature. The water samples were centrifuged at 4700 x *g* for 10 min at 4 °C to pellet the silica matrix containing the bound eDNA. The silica matrix was resuspended in 500 µl of wash buffer (50% [v/v] ethanol, 10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA), transferred to a microcentrifuge tube and washed three times with wash buffer as mentioned above [22]. The supernatant was removed after the final wash, the silica matrix was dried at 70 °C for 30 s and resuspended in 30 µl of nuclease free water followed by incubation at 70 °C for 2 min to elute the bound eDNA. The samples were centrifuged at 11,000 x *g* for 2 min and eDNA was transferred to a fresh tube and stored at -20 °C.

2.6 Conventional PCR

Conventional PCR (cPCR) was performed to validate the eDNA extraction from the water samples using primers specific for the ITS-2 region of *G. truncatula* (FP: CGTTGTCCGTTCATCTCG; RP: CCTGTTCTCCACCCACG). The PCR reaction was performed in a 25 µL reaction with 2x Super Master Mix (Bimake, China) using a T100 Thermal Cycler (Bio-Rad, California, United States). Reaction conditions included an initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s with a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis in a 2% (w/v) agarose gel prepared with 1x TAE buffer (40 mM Tris-HCl pH 8.0, 20 mM acetic acid, 1 mM EDTA) at 100 V for 40 minutes and the gel was stained

with SYBR® Safe DNA Gel Stain following the manufacturer's instructions (Invitrogen). PCR amplicons were visualised on a Gel Doc™ EZ imager (Bio-Rad) using Image Lab™ software (Bio-Rad).

2.7 Quantitative PCR

Quantitative PCR was performed in a Magnetic Induction Cycler (MIC qPCR cycler, Biomolecular systems, Queensland, Australia). The multiplex qPCR method, primers and probes to detect liver fluke and snail were described by Rathinasamy et al. (2018). Briefly, the qPCR assays were performed in triplicate using SensiMix II probe kit (Bioline, Australia). Each 25 µl reaction contains 1x SensiMix II probe mastermix, 300 nM of *F. hepatica* and *A. tomentosa* primers, 100 nM of *F. hepatica* probe, 150 nM of *A. tomentosa* probe, 1 mM $MgCl_2$ and template DNA. MIC qPCR cycling conditions included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C at 10 sec and 60 °C at 20 sec. Each assay contained a positive control (genomic DNA from liver fluke and snail) to confirm the assay reproducibility and no template controls to ensure the absence of reagent contamination.

To assess any inhibition of the qPCR by inhibitors in field samples, 10-fold dilutions of *F. hepatica* (amounts ranging from 14 ng to 14 pg) and *A. tomentosa* (amounts ranging from 50 ng to 0.5 pg) genomic DNA were spiked with eDNA isolated from two field collected water samples that were established to contain no traces of liver fluke or snail eDNA by qPCR and conventional PCR. The standard curves for *F. hepatica* and *A. tomentosa* were generated by plotting the log DNA concentration against the average Ct value obtained in the qPCR assay. Genomic DNA isolated from liver fluke and snail were used as positive controls in qPCR assays.

2.8 Data analysis

The qPCR data from each run was analysed using batch analysis with standard curve generated using genomic DNA of snails and liver fluke spiked with eDNA samples isolated from field collected water samples. The raw cycle threshold (Ct) values were exported from MIC qPCR cycler and analysed using Microsoft Excel (2016). The Logarithm (base 10) of DNA concentration was plotted against the average Ct (Cq) of each concentration to obtain a linear regression line of least square fit and used as a standard curve. The concentration of DNA in unknown samples was calculated using the formula, quantity = (Cq-b)/m where b is y-intercept and m is the slope of linear regression. The difference in the eDNA concentrations among different sample dates were analysed using a one-way ANOVA. The statistical analysis was performed using GraphPad Prism version 7 for Windows, GraphPad Software, La Jolla, California, USA.

3. Results

3.1 Multiplex qPCR detects and differentiates eDNA released from snails and free-living liver fluke stages in irrigation water

The multiplex qPCR assay used in this study has higher sensitivity and specificity to detect snail and liver fluke DNA and the detection limit of the assay to detect genomic DNA from snail and liver fluke has been established to be 50 fg and 14 fg, respectively [17]. To ascertain the detection limits of the assay in the presence of potential inhibitors in eDNA isolated from field samples, we tested multiple 10-fold dilutions of snail and liver fluke genomic DNA spiked with eDNA samples (1:20 dilution) negative for liver fluke and snail eDNA in the multiplex qPCR assay. We observed a linear standard curve for detection of DNA of *A. tomentosa* and *F. hepatica* in the presence of field collected eDNA samples suggesting no or minimal inhibition from the field collected water samples (Fig. 2; Supplementary Table 1). We noticed a minimal inhibitory effect on the reaction efficiency of primer sets used in the multiplex qPCR assay from potential inhibitors in the eDNA from field collected water samples. The reaction efficiency of snail specific primer has reduced from 90% (normal reaction efficiency) to 75–80% in the presence eDNA from field samples. Similarly, liver fluke specific primer sets showed a reaction efficiency 96% with genomic DNA and 90–107% in the presence of eDNA from field collected water samples. The minimal inhibition of reaction efficiency in snail specific primer sets has translated in the reduction of detection limit to 50 pg in presence of eDNA from field collected water sample as opposed to 50 fg for genomic DNA. Interestingly, we observed no changes in the detection limit for live fluke specific primer set in presence of eDNA from field collected water samples. Furthermore, we assessed the specificity of the assay to amplify the ITS-2 region specific for *A. tomentosa* and *F. hepatica* from the field collections in a conventional PCR using randomly selected positive samples (n = 2) from September 2016. The ITS-2 region of liver fluke and snail eDNA was detected and sequencing confirmed the PCR products, showing the specificity of the assay (data not shown).

3.2 EDNA released from snails and liver fluke stages show seasonal variation in irrigation water on a farm.

To demonstrate the proof of concept for using eDNA approach for large scale monitoring of snail and free-living liver fluke stages in water bodies, we collected a total of 68 water samples from an irrigation channel on a farm over an 11 -month period from February 2016 to December 2016. We selected the study farm based on ongoing reports of liver fluke infection in the farm and we have further identified active liver fluke infection in the farm during selected sample collection dates (Supplementary Table 2). Our search for snails (physical identification) in the irrigation channel during water sample collection failed to identify any liver fluke transmitting snails, however, we have identified multiple non-liver fluke transmitting snails (Table 1). Prior to the analysis of eDNA isolated from irrigation water in multiplex qPCR assay, we checked the amplification of amplification of *G. truncatula* ITS-2 using conventional PCR and we observed positive amplification of *G. truncatula* ITS-2 in all the samples, confirming the eDNA isolation.

Table 1
Identification of snails in water sample collection sites in the irrigation channel in study farm at Maffra, Victoria, South-east Australia.

Sampling date	No. of snails identified in physical observation		Liver fluke transmitting snail eDNA detection	Remarks
	Liver fluke transmitting snails	Non liver fluke transmitting snails		
February 2016	0	64	4/11	Slow water flow
March 2016	0	30	11/11	Average water temp 15˚C, recently irrigated, water quite deep
May 2016	0	23	9/11	Average Water temp 11˚C, shallow water, no flow
September 2016	0	20	11/11	Average water temp 15˚C, slow water flow
October 2016	0	6	3/7	Average water temp 14˚C, slow water flow
November 2016	0	4	0/6	Average water temp 18.5˚C, no water flow
December 2016	0	7	5/11	Average water temp 13˚C, Irriragation in process, water flowing

We tested eDNA isolated from irrigation water samples in multiple qPCR and quantified the eDNA levels using the standard curve described above. The cut off Ct value for liver fluke and snail eDNA detection was set at 38 and any sample providing a Ct value above 38 was considered to be negative for liver fluke or snail eDNA. We have identified liver fluke specific eDNA in 56/68 sites from all seven time points analysed in this study and snail specific eDNA was identified in 44/68 sites analysed in this study and no snail specific eDNA was identified in sample collection sites in November 2016 (Fig. 3). Generally, we observed a trend of higher levels of snail eDNA relative to liver fluke eDNA at all the time points analysed as expected due to the large size of the snail and the higher potential for eDNA release. The quantity of eDNA specific for snail and liver fluke varied over the 11-month period, demonstrating the change in dynamics of eDNA in irrigation water. For liver fluke, eDNA levels varied at different sites along the channel as well as at different times during the year: the highest liver fluke eDNA levels were observed in late Summer (February-March) and early Spring (September). For *A. tomentosa*, eDNA levels also varied at different sites along the channel as well as at different times during the year, with the highest levels observed in March, September and December (early summer). The eDNA levels of *F. hepatica* or *A. tomentosa* were significantly different among the different site samples collected in the same month ($p <$

0.05, Fig. 3). However, the variations in mean snail and fluke eDNA levels, respectively, across the time points were not significant.

4. Discussion

Molecular assays for eDNA detection allow for effective identification of parasites stages or intermediate hosts in water bodies paving the way for large scale monitoring of parasite transmission dynamics in short time. EDNA approach for parasite identification has been demonstrated for identification eDNA released from liver fluke and other parasitic trematodes such as *Opisthorchis viverrini*, *Calicophoron daubneyi* and *Ribeiroia ondatrae* has been successfully detected in PCR based assays [23, 24, 25]. QPCR based identification of eDNA released from liver fluke or liver fluke transmitting snails in the field is a promising option to monitor fluke and snail prevalence, given the superior sensitivity, quantitative nature and cost effectiveness of qPCR assays [15, 25].In this study, we have successfully applied a multiplex qPCR assay to detect and quantify eDNA of *F. hepatica* and *A. tomentosa* snail in irrigation water samples from a farm in Maffra, Victoria, South-east Australia (Rathinasamy et al., 2018). We have assessed the specificity of multiplex qPCR to detect *F. hepatica* and *A. tomentosa* against DNA from other common helminth parasites of livestock and other snail species, respectively [17]. However, the specificity of this assay against other trematode infections of *A. tomentosa* including causative agents of avian schistosomiasis have not been performed and sequence information for some of the trematodes infecting *A. tomentosa* are not available in the public domain.

We have used a small volume of water sample (triplicate sample of 10 ml water for each sample) for eDNA analysis in this study as opposed to large volume of samples (200–500 ml) used for eDNA detection in other studies [26]. We have used 10 ml of water sample to avoid co purification of inhibitory substance as it is a common limitation when using large volume of water samples and small volume of water samples (15–50 ml) have been successfully used for detect eDNA released from snakes and turtles [27, 28, 29]. Furthermore, we have experienced PCR inhibition from humic substances co purified with eDNA from large irrigation water samples (unpublished). During the study period, the eDNA levels of or *A. tomentosa* or *F. hepatica* in water samples ranged from 0.32–358 pg and 0.10-16.26 pg, respectively (Fig. 3). Differential levels of both snail and liver fluke eDNA were observed among the samples analysed in this study, suggesting variation in the abundance of both *A. tomentosa* and the liver fluke parasite in water bodies. Peak levels of liver fluke DNA occurred in late summer (February, March) and early Spring (September) whereas peak levels of snail eDNA were observed in late summer (March), early Spring (September) and early summer (December). The incidence of fluke infections in *A. tomentosa* in an irrigated district (Griffith, N.S.W., South east Australia) was reported by Boray et al (1969) to peak in Spring (September-November) which is generally consistent with the peak of liver fluke eDNA we observed in September. The levels of *A. tomentosa* in the Central Tablelands of N.S.W. (South-east Australia) were assessed by Boray (1969b) to peak in February-May and December which is also generally consistent with the peaks of snail eDNA we observed. However, further experiments are required to correlate the qPCR signal to the actual snail biomass in water bodies.

The eDNA levels of mud snails, fish or amphibians were shown to correlate with known organism density or biomass of the organism in controlled mesocosm studies [30, 31, 32]. The eDNA levels of several species (amphibians, fish and aquatic heteropteran) in field samples were shown to correlate with density of the organism [33, 34, 35]. Further, the lower levels of eDNA observed in certain samples in this study could be attributed to factors influencing the formation and decay rates of liver fluke or snail eDNA released in water bodies. For instance, eDNA shedding levels from snails could be dependent on snail size, nutrition status, temperature of the water and liver fluke infection levels while the decay rate of eDNA could be influenced by temperature, light exposure, salinity, microorganisms and water flow [36].

Identification of liver fluke eDNA in the absence of snail eDNA suggests an alternative source for liver fluke eDNA such as eggs or adult fluke DNA released in the faecal samples contributed to liver fluke eDNA as the cattle graze in the vicinity of the irrigation channel. Furthermore, rainwater overflow into the irrigation channel from the paddock could potentially introduce faecal eDNA and alter eDNA profiles. The irrigation channel has been fenced on both sides and cattle gaining access to the irrigation channel is rare, but it cannot be ruled out completely. The current method of liver fluke eDNA identification lacks the ability to differentiate the source of eDNA as it could be from miracidia, cercariae, metacercariae or adult fluke DNA released in faeces of infected animals. However, this limitation can potentially be overcome by using an environmental RNA (eRNA) approach where transcript sequences specifically expressed in certain life stages (i.e. miracidia, cercariae or metacercariae) can be used as a target in PCR assays to ascertain the liver fluke life stage present in the water samples (Jones et al., 2018).

5. Conclusions

We have demonstrated a proof of concept for detection and quantification of eDNA of liver fluke transmitting snails and free-living liver fluke life stages using multiplex qPCR assay. However, this study is only an initial step in using eDNA approach to understand liver fluke transmission dynamics on farms and provided valuable, albeit limited data, to monitor spatial variations of eDNA levels. Further development and application of this assay to assess the presence and levels of liver fluke transmitting snails and free-living liver fluke stages in the water bodies on irrigated farms will potentially allow producers and local authorities to estimate the risk of liver fluke exposure on farms. These data could be pivotal to implementing specific integrated parasite management plans to minimise production losses caused by liver fluke. In the future, this assay can also potentially be utilised to map the snail prevalence in water bodies and potentially develop a snail control program.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The information supporting the conclusions of this article is included in the article.

Competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors' contributions

VR, JS, LT, JK, CH, GW, MK nad TE was responsible for the experimental work, data analysis, and writing of the manuscript. TS, GR and TB coordinated the project, contributed to the experimental design, and reviewed the drafts. All authors read and approved the final version of the manuscript.

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Figures

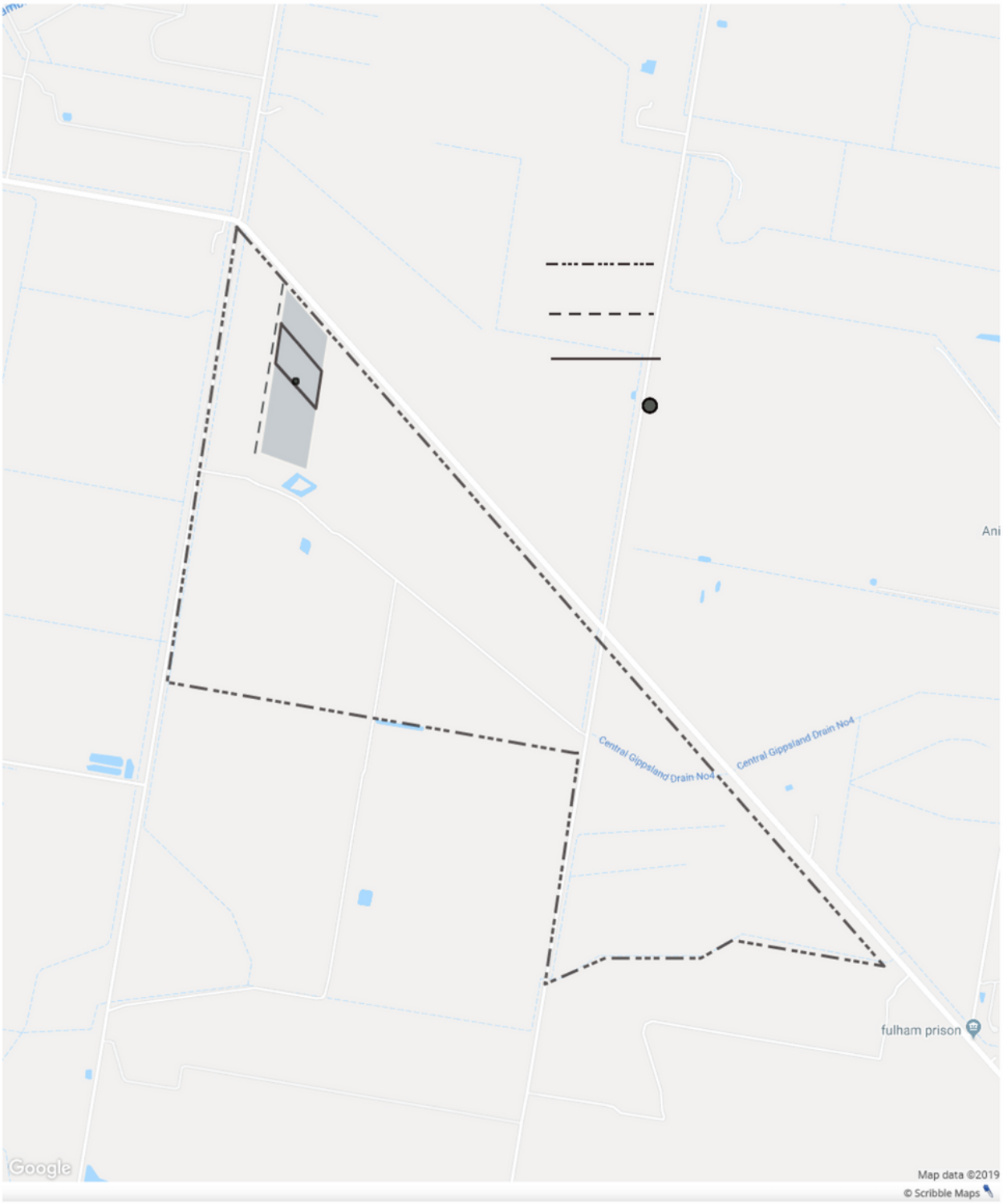

Figure 1

Map of the study farm and irrigation channel used in sample collection.

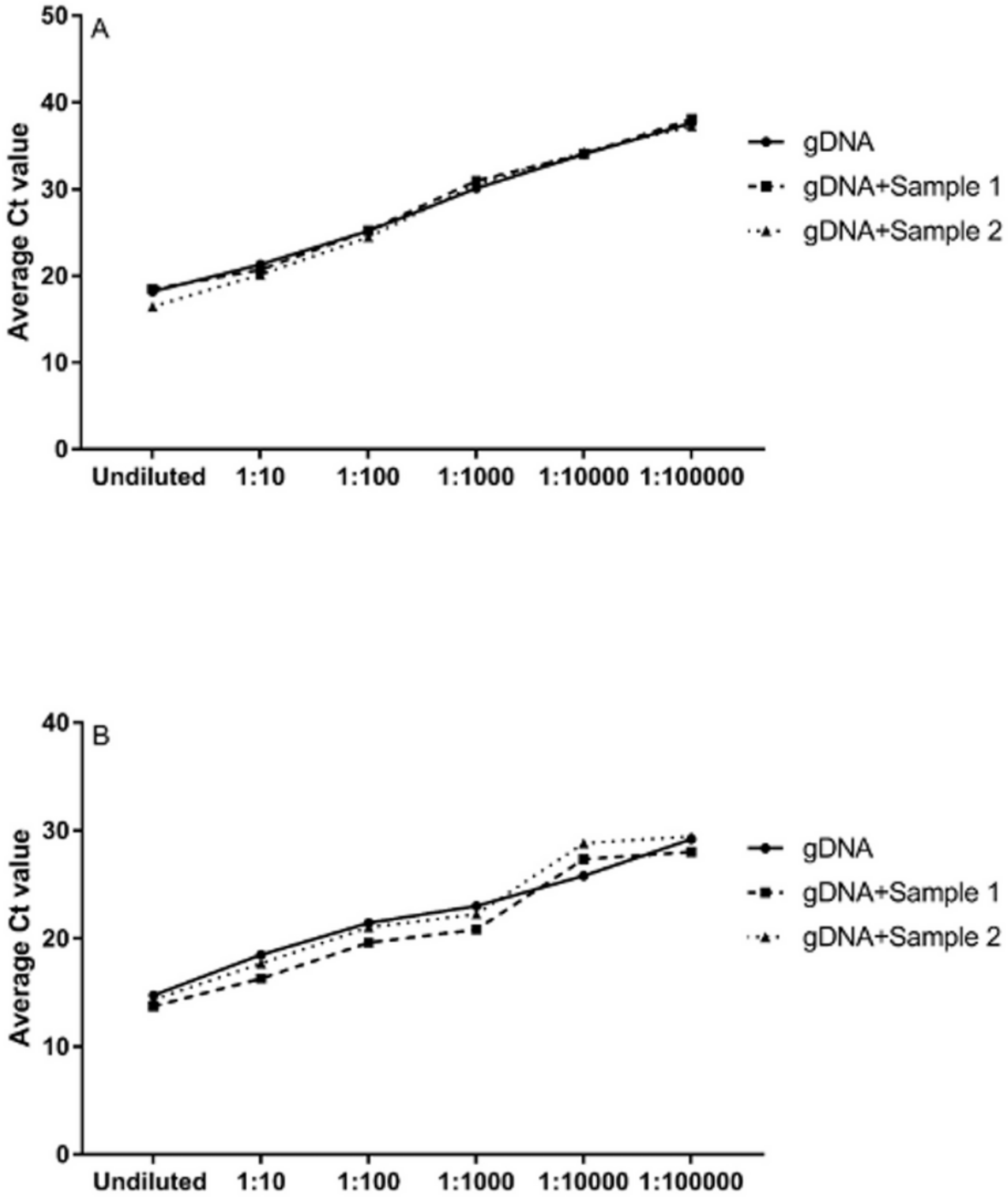

Figure 2

Quantitative PCR based amplification of ITS-2 from genomic DNA of Austropeplea tomentosa or Fasciola hepatica spiked with eDNA isolated from field collected water samples. Multiple dilutions (10-fold dilutions) of Austropeplea tomentosa genomic DNA (A) and Fasciola hepatica genomic DNA (B) were analysed alone or mixed with eDNA isolated from field collected water samples (sample 1 and sample 2) in a multiplex qPCR assay. The average Ct values were plotted against the DNA dilutions in the graph and

average Ct values for liver fluke or snails obtained from multiplex qPCR assay were separated into separate graphs. Each data point represents the mean of three replicates.

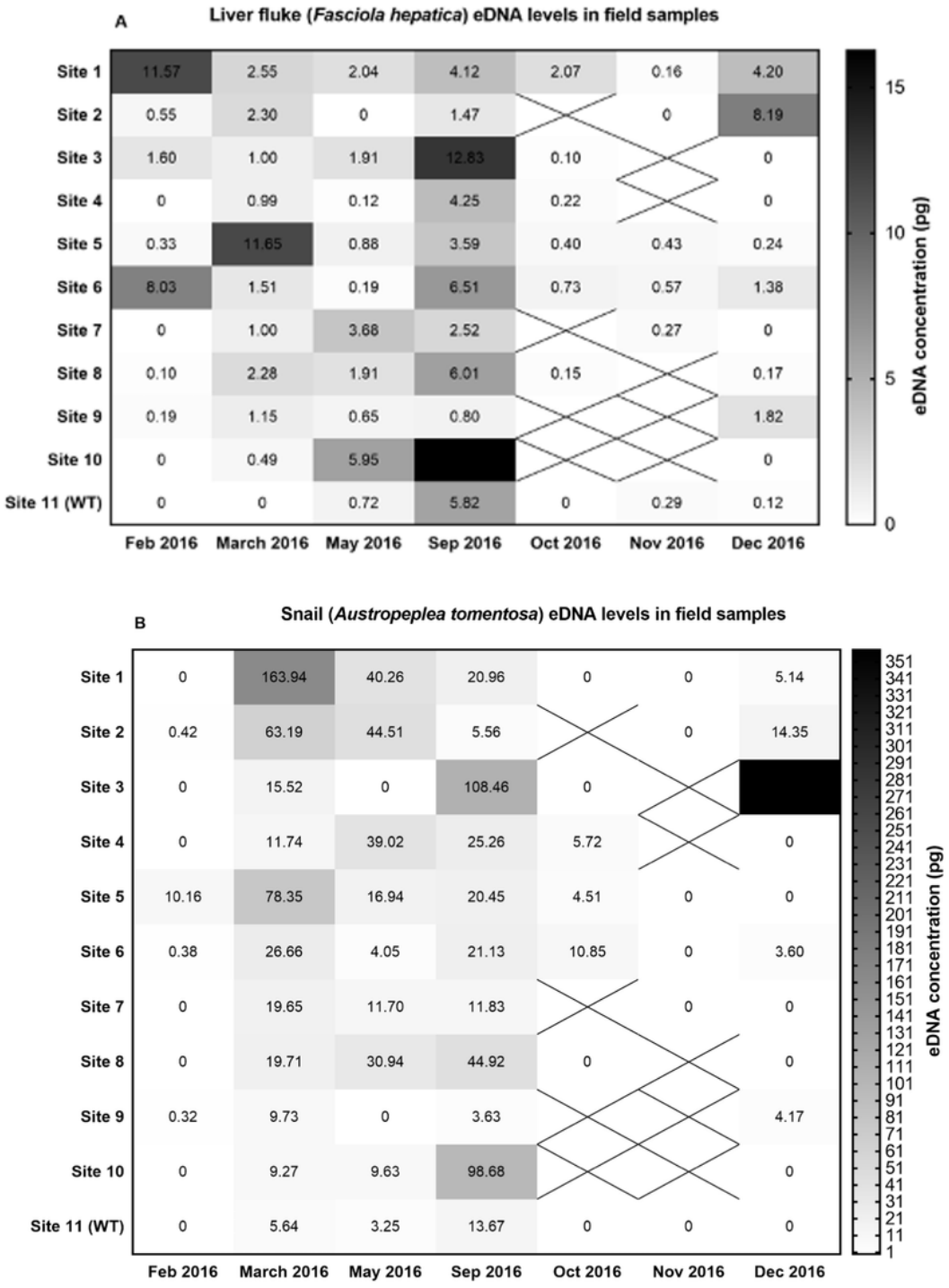

Figure 3

Assessment of eDNA levels of Fasciola hepatica (A) and Austropeplea tomentosa (B) and in water samples collected over an 11-month period from a farming property at Maffra, Victoria, Australia. The eDNA isolated from water samples was analysed using the multiplex qPCR assay with a mix of

Austropeplea tomentosa and Fasciola hepatica genomic DNA as a positive control (data not shown). Each sample was analysed in triplicate and the mean Ct value for Austropeplea tomentosa and Fasciola hepatica from each sample were used to calculate the concentration of eDNA (pg). Each site in the channel is separated by approximately 10 metres. Site 11 (WT) is the water trough. The cross hatched indicates no water sample available at that site.