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Development and use of a nucleic acid-based assay to determine the natural and experimental
prevalence of Fasciola hepatica in lymnaeid intermediate hosts
by Kristi Leigh Dimke

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Abstract:
Fasciola hepatica is an increasing problem for livestock producers in Montana and elsewhere. A
thorough understanding of the interaction of the fluke with its intermediate snail host is necessary to
implement pest control measures and reduce risk to livestock. To this end a sensitive, specific nucleic
acid based assay was developed which detects fluke material in snails immediately after they become
infected and throughout the course of infection in the snail. The assay involves isolating and purifying
RNA from snails, making cDNA using reverse transcriptase, and using a fluke specific primer to
amplify a fluke sequence on the small ribosomal subunit. After separation, by gel electrophoresis and
Southern blotting, an end-labeled fluke specific probe is used to detect the fluke sequence. The assay
was then used to determine the natural prevalence of F. hepatica infection in an enzootic area in
Montana over a three year period. One Lymnaea modicella snail was found naturally infected during
the study in the month of August, supporting the hypothesis that disease transmission occurs in the fall
in this region of the U.S., however, the presence of fluke-positive snails at a neighboring thermal spring
indicate that disease transmission may occur over a longer season at these areas. Experimental
infections of several species of lymnaeid snails reveal that L. bulimoides and L. modicella are the most
important intermediate host snails in Montana. Although all other species tested acquire the infection in
large numbers, most do not routinely carry the infection to patency; in fact, a large percentage
overcome the infection by 5 days post exposure; which may suggest the presence of a molluscan
internal defense mechanism.

DEVELOPMENT AND USE OF A NUCLEIC ACID-BASED ASSAY TO

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APPROVAL

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Kristi Leigh Dimke

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Fasciola hepatica is an increasing problem for livestock producers in Montana and elsewhere. A thorough understanding of the interaction of the fluke with its intermediate snail host is necessary to implement pest control measures and reduce risk to livestock. To this end a sensitive, specific nucleic acid based assay was developed which detects fluke material in snails immediately after they become infected and throughout the course of infection in the snail. The assay involves isolating and purifying RNA from snails, making cDNA using reverse transcriptase, and using a fluke specific primer to amplify a fluke sequence on the small ribosomal subunit. After separation by gel electrophoresis and Southern blotting, an end-labeled fluke specific probe is used to detect the fluke sequence. The assay was then used to determine the natural prevalence of F. hepatica infection in an enzootic area in Montana over a three year period. One Lymnaea modicella snail was found naturally infected during the study in the month of August, supporting the hypothesis that disease transmission occurs in the fall in this region of the U.S., however, the presence of fluke-positive snails at a neighboring thermal spring indicate that disease transmission may occur over a longer season at these areas. Experimental infections of several species of lymnaeid snails reveal that L. bulimoides and L. modicella are the most important intermediate host snails in Montana. Although all other species tested acquire the infection in large numbers, most do not routinely carry the infection to patency; in fact, a large percentage overcome the infection by 5 days post exposure; which may suggest the presence of a molluscan internal defense mechanism.

INTRODUCTION

Historical and Economic Significance

Fasciola hepatica, the common liver fluke, is a parasite of worldwide importance,

affecting cattle and sheep most commonly, but also goats, non-domestic animals, and

humans. These digenetic trematodes are acquired by the definitive host by ingestion of the

infective stage, the metacercaria, which is encysted on some type of vegetation.

Excystment occurs in the digestive tract of the host, and immature flukes penetrate

through the wall of the small intestine, find their way to the liver and penetrate the liver

capsule. For the next six weeks the flukes migrate through the liver and finally come to

rest in the bile ducts where they begin to produce eggs. The eggs, which are liberated in

the feces of the infected animal, hatch in two weeks under ideal circumstances and the

free-swimming miracidia must then find a suitable snail intermediate host (snail of the

genus Lymnaea) before their energy reserve is exhausted. When a suitable lymnaeid snail

is encountered, the miracidia penetrate into the snail and form sporocysts, which then give

rise asexually to many rediae. These rediae give rise to tailed cercariae which leave the

snail to encyst on vegetation and begin the cycle anew. The disease, fasciolosis, takes a

chronic course in the bovine host, causing production loss and condemnation of the liver

at slaughter, while in the ovine host the disease is often acute, resulting in death if not

treated promptly. Since the original description of the fluke by Jean de Brie in 1379,

(Taylor, 1964), records show that epidemics have plagued Europe regularly, wiping out

millions of sheep. The disease has now spread to nearly every continent, and there is

potential for disease transmission wherever infected animals come into close proximity

with an appropriate snail vector (Knapp et al., 1992). Worldwide losses due to fasciolosis

are estimated at 2 billion dollars annually, and the economic impact will continue to be felt

as the growing world population puts additional demands on agriculture to produce more

high quality food products, such as animal protein (Boray, 1994). Additionally, in recent

times fasciolosis has been recognized as an important world human health problem,

especially in France and South American countries like Bolivia, where it has been reported

that 40% of the population has been exposed (Hillyer, 1994).

In the United States, the disease is present in the West-Coast and Rocky Mountain

states, and the Southwestern, Midwestern, and Gulf-Coast states. Krull (1934) reported

that there were suitable snail vectors present in every state in the U.S., and concluded that

the spread of disease to every state was possible. In addition, the incidence of the disease

has been rising, from a widely accepted figure of about 5% reported in the early 1970's

(Foreyt and Todd, 1976) to 17-19% in the 1990's, with some areas reporting almost 50%

prevalence in slaughter cattle (Knapp et al., 1992, Briskey et al., 1994). Since the U.S. is

a major world food producer, this disease is likely to have increasing economic impact in

the future.

With half a million sheep and over 2.4 million cattle, Montana is one state likely to

feel a heavy economic impact from this disease. A survey done in 1992 by Knapp et al.

reported the incidence of disease among Montana slaughter cattle to be 17.24%, up from

5% in 1976 (Foreyt and Todd, 1976). A survey of lymnaeid snail distribution in Montana

done between 1990 and 1992 found that the snails are present in at least 28 of the 56

counties in Montana, and so the spread of the disease to these 28 counties is a distinct

possibility (Dunkel et al., 1996).

Conventional Methods of Control

Previously, chemical control methods including treatment of the definitive host

with anthelmintics and eradication of the intermediate host with molluscicides were heavily

relied upon to keep fasciolosis in check. Hexachloroethane and carbon tetrachloride were

widely used as anthelmintics until the 1970's, when concern over the buildup of

carcinogenic agents in meat intended for human consumption led to restrictions on their

use (Malone et al., 1982). Anthelmintics in use today which are effective against mature

flukes include the salicylanilides (rafoxanide, closantel), halogenated phenols (nitrooxynil,

bithionol), and benzimidazoles (triclabendazole, albendazole, mebendazole) (Boray, 1994).

The only anthelmintic which is highly effective against both mature and immature flukes is

Clorsulon. In some areas reports indicate that flukes are becoming resistant to these

agents, and treatment with two different anthelmintics is recommended (Boray, 1994).

Unfortunately, the prospects for development of new anthelmintics are limited (Boray,

1994), necessitating the judicious use of the ones available. Several molluscicides,

including copper sulfate, niclosamide, and n-trityl morpholine (Frescon) have been widely

used to control the snail intermediate host and therefore the spread of the disease

(Chroustova and Willometzer, 1974, Lam et al., 1989, and Pantelouris, 1965). These

efforts have been fairly successful, especially when combined with anthelmintic treatment

of the definitive host (Chroustova and Willometzer, 1974). It has been suggested,

however, that the risk of resistance developing in the target species is present whenever

control involves long term use of pesticide, and indeed, increasing resistance to

molluscicides has been reported among some trematode intermediate host snail species

(Lam et al., 1989). In addition, environmental regulations have ended the use of

molluscicides in the U.S., and other countries are reevaluating the risks associated with

their continued use. Recently, the isolation from plants of natural products with

molluscicidal properties has received attention, however, this research is still in the early

stages (Singh et al., 1993, and Appleton et al., 1992).

Other options for control of the snail intermediate host include drainage of snail

habitat and introduction of snail predators and competitor snails. With regard to drainage

of snail habitat, snails are capable of aestivating for at least a year (Kendall, 1950), and so

may seem to disappear only to appear again with the return of moisture. For this reason,

drainage of snail habitat is impractical as a means of controlling snail populations,

especially on farmland where irrigation is necessary. Most of the research done on the use

of snail predators and competitor snails has been done to try to control the snail

intermediate hosts of Schistosoma mansoni and other medically important trematodes. For

schistosome snails, several potential competitor/predator organisms have been

investigated, including North American crayfish, sciomyzid flies, and several species of

snails including Melanoides tuberculata and Heliosoma spp. (Hofkin et al., 1991, Appleton

et al., 1993, and Kruatrachue and Upatham, 1993,). Efforts to find a similar means of

control for F. hepatica intermediate hosts have not been as promising. The remarkable reproductive potential and hermaphroditic nature of these snails makes their eradication virtually impossible.

In the future, we need to be increasingly broad-based and innovative about developing control measures for fasciolosis. In his 1993 address to the American Society of Parasitologists, K. Darwin Murrell outlined an integrated pest control plan, which included the strategic use of anthelmintics based on an epidemiological understanding of parasite transmission, enhancement of host resistance (either the intermediate or definitive host) through breeding or vaccines, biological control, and the implementation of management practices designed to reduce the risk of parasite transmission (Murrell, 1994). All of these options necessitate a thorough understanding of parasite transmission, in which the snail intermediate host plays a pivotal role. Indeed, any of the above mentioned control measures could be applied to the intermediate host. It is therefore necessary to give attention to identifying the intermediate host snails in given areas, factors which influence their life cycle, and factors which influence the snail-parasite interaction in order to develop new management and control practices.

Seasonal Transmission Studies in the U.S.

To identify potential intermediate host snail species, high risk areas, and peak seasons for disease transmission, snail and seasonal transmission studies have been done in various parts of the U.S. Five species of lymnaeid snails have been identified in Oregon,

and Lymnaea bulimoides has been present in every fluke infested area (Shaw and Simms, 1929). Lymnaea bulimoides has been reported from Texas (Olsen, 1944), and a study done in 1971 by Wilson and Samson found L. bulimoides, L. palustris, and L. modicella present at the junction between Colorado, New Mexico and Arizona; where the infection rate among cattle and sheep was 30% and 49% respectively. In Louisiana, where the infection rate in cattle reaches 80%, L. bulimoides is the principal lymnaeid snail present (Lindsay, 1979). Further study in Louisiana has shown that disease transmission occurs primarily in spring and early summer, and furthermore, that the risk of disease transmission can be predicted based on the numbers of L. bulimoides, which can be correlated to microclimatic conditions (Malone et al., 1982 and Malone et al., 1984). Disease transmission studies conducted in Florida using tracer sheep indicate that transmission peaks in February and April, ceases in the summer, and resumes again in November, December and January (Boyce and Courtney, 1990). In Oklahoma, which was not thought to have any incidence of indigenous infections, the presence of lymnaeid snails was confirmed, as were several cases of fasciolosis in native cattle, indicating the occurrence of disease transmission in the state (Cheriuyot and Jordan, 1990). In Western Montana, where up to 90% incidence of liver fluke infection has been reported in slaughter cattle (Marley et al., 1994), L. modicella is the predominant intermediate host snail species present (Dunkel et al., 1996).

Studies of Snail-Parasite Interaction

The last piece of the life cycle of F. hepatica to be elucidated was that a snail is the

intermediate host. This was confirmed in 1857 by Wagener who observed the penetration

of the miracidium into the snail and its development into rediae (Taylor, 1964). That a

lymnaeid snail is the intermediate host for F. hepatica was proven in 1882 by Rudolf

Leuckart (Taylor, 1964) and independently by Sir Algernon Thomas, who found naturally

infected L. truncatula and produced fasciolosis in rabbits with metacercariae derived from

these snails (Thomas, 1883). Lymnaea truncatula, which is not present in the U.S., has

since been shown to be extremely susceptible and is the main intermediate host in Europe,

as natural infection rates among these snails are 100% in some areas (Ross and O'Hagan,

1968). In the U.S., the first reports of naturally infected snails were of L. bulimoides in

1929 in Oregon (Shaw and Simms, 1929), and in Louisiana (Sinitsin, 1933). Since then,

several more have reported finding naturally infected L. bulimoides in Texas, Colorado,

Washington, and Louisiana respectively (Olsen, 1944, Wilson and Samson, 1971, Lang,

1977, and Lindsay, 1979), however, with extremely low infection rates, averaging less

than 0.5%. In 1977, Lang also reported finding naturally infected L. palustris, L. proxima,

L. stagnalis, and L. modicella in Eastern Washington, although the infection rate was not

given. At the end of a three year survey in Louisiana, Malone et al., (1984) reported

finding two naturally infected snails, both of the genus Lymnaea. Due to the difficulty in

finding naturally infected snails, experimental exposure of snails to miracidia of F. hepatica

has become the method of choice to study the snail parasite interaction and to identify possible intermediate host species. Additional possible intermediate host species identified by successful experimental infection include L. columella, L. modicella, and L. traskii (Krull, 1933a, 1933b, 1934); and L. montanensis (Rowan et al., 1966) and L. cubensis (Cruz-Reyes and Malek, 1987).

Other attempts at experimental infection of snails have been less successful. Kendall (1949) exposed 101 L. stagnalis to F. hepatica miracidia, but only 13 of those became infected. Boray (1966) experimentally infected L. stagnalis, L. palustris, L. peregra, L. auricularia, L. truncatula, L. tomentosa, and L. lessoni, however, only L. truncatula and L. tomentosa were susceptible as adults, the rest were only susceptible when immature. Lymnaea lessoni and L. auricularia never produced cercariae. Experimental exposures done by Wilson and Samson (1971) on L. modicella and L. palustris indicated that the snails were susceptible only when less than 30 days old, and Rowan et al., (1966) found the same to be true for L. montanensis. Foreyt and Todd (1978) exposed L. bulimoides, L. caperata, L. modicella, L. umbrosa, L. palustris, and L. stagnalis to F. hepatica miracidia but failed to infect any but L. bulimoides. Bouix-Busson et al., (1984) found that 70% of juvenile and 95% of adult L. glabra develop abortive infections when exposed to F. hepatica, whereas the normal host, L. truncatula, develops evolutive infections 80% of the time, whether juvenile or adult. These results have led to the study of differential susceptibility among lymnaeid snail species.

Although many studies have been done to identify the external stimuli which attract miracidia to their snail hosts; the observation that infection rates and outcomes are

age dependent as well as species dependent has led to the search for a snail internal defense mechanism which could be responsible for terminating the development of the parasite. To address this issue, Boray (1966) transplanted F. hepatica rediae into adult L. stagnalis to see whether they would continue to develop, and found that they were destroyed by a host tissue reaction. Similarly, histological examination of juvenile L. palustris exposed to F. hepatica showed that all sporocysts were removed by a cellular encapsulation response in less than 14 days (McReath et al., 1982). Further, lymnaeid snails have been shown to have phagocytic cells, termed hemocytes, circulating in their hemolymph which are capable of encapsulating and destroying a variety of foreign particles. It remains to be discovered why some species of lymnaeid snails are able to destroy invading F. hepatica sporocysts through a hemocyte mediated encapsulation response and others, while possessing morphologically similar hemocytes, are not.

Although the range and distribution of the disease and the potential snail intermediate hosts in Montana have been identified (Knapp et al., 1992 and Dunkel et al., 1996, respectively), there is a lack of information regarding these snails and factors affecting the snail-parasite interaction which in turn have a crucial impact on the seasonal transmission cycle and ultimate risk to Montana livestock. It is therefore important that the snail intermediate host species be positively identified and the intermediate host potential of all lymnaeid snail species present in Montana be assessed. In order to accomplish this, new methods must be developed to detect naturally infected snails and to conveniently assess the susceptibility characteristics of each.

Our work on fasciolosis began in 1989 with a Montana Agriculture Experiment Station project designed to investigate the epizootiology of fasciolosis in Montana livestock and to determine ways in which the disease might be controlled. The work contained in this thesis is the result of two of the objectives outlined in the proposal, the first of which was to develop a DNA probe whereby minute quantities of the liver fluke may be detected in the snail intermediate host and thereby permit early detection of infected snail populations; and second, to determine the identification and distribution of the snail intermediate hosts for F. hepatica in Montana.

DEVELOPMENT AND TESTING OF A NUCLEIC ACID-BASED ASSAY TO DETECT FASCIOLA HEPATICA INFECTED INTERMEDIATE HOSTS

Introduction

The development of a better method of detection of infected snails was a necessary first step in the study of the snail host and the snail parasite interaction. Several procedures are commonly used to determine if a snail is infected with F. hepatica. Collected snails are observed microscopically to determine whether they are discharging cercariae or crushed and examined microscopically to determine whether there are larval forms of the parasite present in the snail tissue. Unfortunately, only mature infections can be detected in this manner, sporocysts are not visible unless the snail is sectioned and stained. Examination of individual snails in this way is time consuming and impractical when large numbers of snails need to be evaluated.

Nucleic acid probes have gained widespread acceptance as useful field diagnostic tools. Probes have been developed for several organisms including Aeromonas salmonicida, Plasmodium berghei, and Tritrichomonas foetus (Barry et al., 1990; Waters and McCutchan, 1990, and Severson, 1991). The approach taken by our group was to make an oligonucleotide probe derived from a hypervariable region of ribosomal RNA sequence from Fasciola hepatica (Shubkin et al., 1992). Because rRNA is present in multiple copies in each cell and contains hypervariable regions which can be used to

differentiate genera and species (Dams et al., 1988), it was a logical target for

development of a probe. The initial oligonucleotide probe developed was used to detect a

fluke specific sequence in a northern blot of total snail RNA (Shubkin et al., 1992).

Subsequently, other groups have developed nucleic acid probes which detect fasciolid

specific repetitive DNA sequences in preparations of snail DNA (Heussler et al., 1993;

Kaplan et al., 1995). All of these methods, however, require preparation and testing of

each snail individually. Our goal was to increase the sensitivity of our procedure so that

snails could be processed in large batches, enabling one person to screen several thousand

snails in a short time.

The first method developed in our lab used an oligonucleotide probe derived from

an rRNA sequence to detect the fluke sequence in a northern blot of total snail RNA

(Shubkin et al., 1992). The oligonucleotide sequence used for the probe was obtained by

amplification from total RNA extracted from whole liver flukes. The primers used in the

reverse transcriptase reaction (RT) and polymerase chain reaction (PCR) were

oligonucleotide primers which hybridize to conserved regions of small subunit rRNAs. A

region of approximately 650 base pairs was amplified, subcloned and sequenced. The

sequence was analyzed and a region unique to Fasciola hepatica was identified

(Figure 1.1.).

This sequence (Figure 1.1.) was used to develop the probe for F. hepatica.

Testing of this probe indicated that it was specific to F. hepatica and that it could detect

fluke material in preparations of total snail RNA; however, five miracidia were required to

give a positive result. Since greater sensitivity was believed necessary to detect fluke

material in batches of snails, a modified method was developed involving PCR

amplification of the fluke sequence before probing.

```
Human 18S      AATGAGCAAT AACAGGTCTG TGATGCCCTT AGATGTCCG        1515
F. hepatica    .......... .......... .......... .........
L. columella   .......... .......... .......... .........

Human 18S      GGC-TGCACG CGCGCTACAC TGACTGGCTC AGCGTG-TG        1552
F. hepatica    ...CC..... T........A ....G.TT.. ....A.TT.
L. columella   ...--..... .......... ...AG.AA.. ......GA.

Human 18S      CCTACCCTAC GCCGGCAGGC  1572
F. hepatica    GAAT..TGG. CTGA.....T
L. columella   ...-....GG C...AA...G
```

Figure 1.1. Sequence alignment of partial DNA sequences for small subunit rRNA in human, Fasciola hepatica and Lymnaea columella. Dashes represent deletions and dots represent sequence identity. Numbering of the human sequence is included for reference (Shubkin, et al., 1992).

This second method used a different region of sequence (Figure 1.2.) for the probe

(called CS 6) and included RT and PCR reactions to amplify the region of sequence

containing the probe site (Rognlie et al., 1994). The primer (called CS 9) for first strand

cDNA synthesis was designed to hybridize to a semiconserved region downstream of the

probe hybridization site. The primer (called CS 11) used in the PCR reaction was

designed to hybridize to a variable sequence region unique to the Fasciolidae so that only

amplification of the fluke sequence would occur.