ROLE OF P-GLYCOPROTEIN IN *HAEMONCHUS CONTORTUS* ANTHELMINTIC RESISTANCE

A Thesis

by

PAMELA DONN GARRETSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2007

Major Subject: Veterinary Parasitology

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Approved as to style and content by:

Chair of Committee:	Patricia Holman
Committee Members:	Thomas Craig
	Clare Gill
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ABSTRACT

Role of P-glycoprotein in *Haemonchus contortus*

Anthelmintic Resistance. (August 2007)

Pamela Donn Garretson, B.S., Colorado State University

Chair of Advisory Committee: Dr. Patricia Holman

The gastrointestinal parasite, *Haemonchus contortus,* is of major concern in the sheep and goat industry as well as in zoological settings. Over the years this parasite has developed resistance to the three classes of anthelmintics, benzimidazoles, imidazothiazoles and macrocyclic lactones, that are currently used for treatment. One of the mechanisms proposed to be involved in this resistance is the efflux transporter P-glycoprotein (Pgp). In this study, the resistance status of several strains of *H. contortus* was evaluated using the larval development assay DrenchRite®. After documenting the resistance status of these strains, transcription of Pgp in L_3 larvae after exposure to anthelmintics was quantitated using polymerase chain reaction (PCR). Of the strains analyzed, only one was determined to be susceptible to all of the anthelmintics tested, while the others showed variable levels of resistance to one or more. A *Haemonchus* strain acquired from a giraffe at a zoo in Florida was the most resistant, showing extremely high levels of resistance to benzimidazoles and levamisole. Molecular characterization of the 18S rRNA gene and the internal transcriber spacer region (ITS) were performed on the giraffe strain to identify the species. Although there were variations in the isolate sequences, the most likely species for the giraffe strain was *H. contortus*. No transcription of Pgp was identified in *H. contortus* L_3 larvae under the

conditions of this study. Thus, increased Pgp does not appear to be a primary mechanism of drug resistance in this stage of the worm.

DEDICATION

I dedicate this to my mother, Mary Lou Welch, who has supported me throughout my life, as well as through this project. She has been an inspiration to me and I would like to thank her for always being there.

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I would like to thank Dr. Patricia Holman for her assistance and guidance throughout this project, not to mention taking on a project involving a parasite that was out of her area of expertise. I think we both learned a lot while working on this project. I would also like to thank Dr. Thomas Craig for his diligent efforts in acquiring parasite strains for use in this study and his extensive knowledge on helminths, especially *H. contortus*. I would like to thank Dr. Clare Gill, who was extremely helpful in the writing process and whose input throughout the project was well appreciated. I would also like to thank Dr. Elizabeth Hammond at Lion Country Safari in Florida for submitting the giraffe fecal sample to the Texas A&M University Diagnostic Parasitology Laboratory for evaluation and allowing us to use the highly resistant strain of *H. contortus* in this study. She has also been extremely helpful in providing background information on how the resistance may have developed in this strain.

I would also like to express my gratitude to my co-workers Eunhee Lee, Dr. Amal El-Gayor, Lauren Lehtinen and Issa Baradji for helping with various aspects of my project and lending a hand when needed. Last of all, I would like to thank my friends and family for always being there when I have needed them and supporting me throughout this study.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

For the sheep and goat industry, the parasitic nematode *Haemonchus contortus* is of great concern. Commonly known as the barber's pole worm, *H. contortus* can inflict a considerable amount of damage to a flock or herd in a short span of time. Haemonchosis, the disease associated with *H. contortus* infections, may be chronic, resulting in minor clinical signs, acute or hyperacute, resulting in death. Treatment and control of this gastrointestinal parasite have been successful through the use of anthelmintics, such as benzimidazoles, levamisole, and ivermectin. However, resistance to these anti-parasitic drugs has developed worldwide and is becoming an area of increasing concern. This has led to an influx of research to determine the mechanisms employed by *H. contortus* that enables them to be highly efficient in drug resistance. By understanding the mechanisms involved, better methods of treatment as well as prevention may be developed.

Haemonchus contortus was first described in 1803 by Rudolphi. The classification is as follows: Class Nematoda, Order Strongylida, and Family Trichostrongylidae. Originally, the parasite was called *Strongylus contortus* and over the years has been referred to as *Strongylus falicollis* (Molin, 1861), *Filaria dendiculata* (Simmonds, 1881) and *Strongylus placei* (Place, 1893) (as cited in Morgan, 1949). It wasn't until the 1900s that *H. contortus* became the preferred nomenclature (Morgan, 1949). There have been several common names associated with *H. contortus*.

This thesis follows the style of Veterinary Parasitology.

These include barber's pole worm, twisted stomach worm, and wire worm (Soulsby, 1982; Kassai, 1999; Anderson, 2000).

The adult female *H. contortus* ranges in size from 2.0-3.5 cm in length and the males from 1.5-2.5 cm (Dunn, 1978). *Haemonchus contortus* are blood sucking parasites that possess a slender dorsal lancet within the buccal cavity for accessing the host blood supply (Soulsby, 1982). The appearance of the female, in which the red, blood filled intestine intertwines with the white reproductive organs, gave rise to the common name barber's pole worm (Morgan, 1949). The males are red in color and possess a characteristic three-lobed copulatory bursa consisting of two symmetrical lateral lobes and one asymmetrical dorsal lobe (Morgan, 1949; Soulsby, 1982). The adults are found in the abomasum, the fourth digestive compartment of the ruminant stomach, where they feed and sexually reproduce.

Haemonchus contortus is distributed throughout the world in tropical and subtropical regions. Approximately 60 different species of ruminants, both domestic and wild, have been identified as hosts for this gastrointestinal worm (Dunn, 1978). Sheep and goats tend to be the preferred host, however, *H. contortus* has been found in cattle, white-tailed deer, bison, antelope, giraffes, and camels to name a few (Hoberg et al., 2004). According to McGhee et al. (1981), cross-transmission is possible between wild and domestic hosts, as was seen in their studies on white-tailed deer and domestic cattle and sheep. Although *H. contortus* has a wide host range, domestic sheep appear to be the most drastically affected, which may be due, in part, to their grazing behaviors. Most sheep exhibit strong flocking habits where they remain in close proximity to each

other while grazing. This concentrates the area of parasite contamination increasing the chance for infection (Dunn, 1978).

Haemonchus contortus has a direct life cycle in the natural hosts. The females are prolific egg layers and can deposit 5,000 to 10,000 eggs per day, which are released into the environment via the host's feces (Morgan, 1949). Once the eggs are released into the environment, larval development occurs and the L_1 stage larvae hatch within 24 hours. The L_1 larvae molt into the L_2 stage larvae soon after and within 3-5 days, molt into the L_3 stage larvae (Veglia, 1916). The resilient L_3 larvae is the infective stage and proceeds to migrate up damp vegetation during early morning and late evening hours in order to increase the chance of ingestion by the ruminant host (Morgan, 1949). This larval migration is highly dependent upon the combination of ideal temperature, humidity and light (Anderson, 2000). Once inside the rumen of the host, the L_3 larvae exsheath, migrate to the abomasum and molt into the L_4 larval stage, followed by the L_5. The L_5 are the immature adults, which develop into mature adults within the abomasum approximately 15 days post infection (Morgan, 1949; Urquhart et al., 1987). Females will begin to produce eggs within 25-35 days post infection (Morgan, 1949).

Warm, rainy weather is essential for the survival and development of the free-living larval stages of *H. contortus* (Kassai, 1999). Larval development is hindered by extremely dry or cold conditions (Kassai, 1999; Anderson, 2000). However, in temperate climates *H. contortus* overcome these adverse conditions through a process called hypobiosis, where the L_4 stage larvae enter into a state of dormancy within the host. Michel (1974) defines hypobiosis as "the temporary cessation of development of

nematodes at a precise point in early parasitic development, where such an interruption contains a facultative element, occurring only in certain hosts, certain circumstances, or at certain times of the year and often affecting only a portion of the worms."

There are at least two types of arrested development that occur in nematodes. The first is termed immune mediated arrest and is considered to be non-specific and of immunological origin. It may arise at any time of the year and be triggered by either host-related or parasite-related factors. The second is termed seasonally induced arrest and occurs at the same time each year. This type of hypobiosis is similar to diapause in insects and is most often triggered by an external environmental stimulus (Horak, 1981; Gibbs, 1986a). The latter appears to be the predominant form of hypobiosis in *H. contortus* (Blitz and Gibbs, 1971; Gibbs, 1986b).

There are three categories of factors that have been suggested as triggers for hypobiosis. These triggers may act independently or in combination with each other to induce an arrested development of the nematode larvae. The first category consists of host-related factors including host resistance, acquired immunity, and age (Blitz and Gibbs, 1972a; Connan, 1975; Horak, 1981; Gibbs, 1982). The second category is parasite-related factors, which include population density and genetic predisposition (Horak, 1981; Gibbs, 1986a). The final category consists of environmental factors including temperature, humidity, and photoperiod length. Since hypobiosis in *H. contortus* appears to be more seasonal than immunological, environmental factors are most likely to be the triggers involved (Blitz and Gibbs, 1972a; Michel, 1974; Horak, 1981; Soulsby, 1982; Gibbs, 1986a, 1986b).

The external triggers for hypobiosis probably act upon the L_3 larval stage in the environment prior to ingestion by the host. These include a decrease in photoperiod from 14.25 to 12.5 hours and an average temperature of 17°C in temperate climates (Blitz and Gibbs, 1972a; Gibbs, 1982). In tropical and arid climates, *H. contortus* may also utilize hypobiosis to withstand hot dry conditions (Gibbs, 1982). However, parasite genetics may also play a significant role in triggering an arrest in development.

Hypobiosis in *H. contortus*, as well as other nematodes, closely resembles the arrested development phenomenon known as diapause in insects. Diapause is an inhibition of development triggered by environmental factors and considered to be genetically controlled. This arrest in development is temporarily irreversible and may continue until either a specific stimulus presents itself or a predetermined period of time has elapsed (Horak, 1981; Sommerville and Davey, 2002).

Once conditions become suitable for parasite survival, the L_4 larvae come out of arrest and continue to develop into the mature adult. By undergoing hypobiosis, *H. contortus* reaches the reproductive stage at a time when it is most beneficial to the parasite and the eggs are released into the environment during conditions that are more favorable for the free-living larval stages (Soulsby, 1982; Urquhart et al., 1987).

There are several factors that may trigger the hypobiotic larvae to emerge from their dormant state and continue development. These include photoperiod, temperature, humidity, and host immune system relaxation due to periparturition and/or lactation. However, the larval development may also recommence spontaneously after a predetermined length of time without the influence of a stimulus (Blitz and Gibb, 1972b;

Horak, 1981; Gibbs, 1982, 1986a). This aspect further demonstrates the similarity between hypobiosis in *H. contortus* and diapause in insects (Horak, 1981).

The resumed development of the arrested larvae often results in the events known as "spring rise" and "periparturient rise." Both are characterized by a sudden marked increase in nematode egg counts as a result of the arrested larvae reaching the reproductive adult stage en masse (Brunsdon, 1964; Procter and Gibbs, 1968). Spring rise, as its name suggests, occurs during the spring and although it is commonly associated with parturition, it is also seen in non-reproducing hosts (Crofton, 1958; Brunsdon, 1964). On the other hand, periparturient rise per se coincides with parturition, and is most evident during the spring lambing/kidding season but may also occur at other times of the year.

The combination of the short life cycle and the survivability of the larvae has enabled *H. contortus* to be a highly infective parasite able to cause a considerable amount of damage to an entire host population. Characteristic clinical signs of a *H. contortus* infection are anemia, edema, bottle jaw (intermandibular edema), lethargy, emaciation, weakness, wool loss, and even death (Dunn, 1978). Anemia is the most common clinical sign resulting from the adult worms feeding on host blood. An adult can consume as much as 0.5 cc of blood in one day (Urquhart et al., 1987). In addition to this, *H. contortus* adults are mobile feeders and move from one feeding site to another, leaving behind wounds that continue to hemorrhage, contributing to the anemia (Soulsby, 1982). The amount of blood that is lost with a high worm burden may result in the death of the host.

The disease associated with *H. contortus* infections is known as haemonchosis, which may be categorized into three forms based on the worm burden and the associated clinical signs. The first, hyperacute haemonchosis, is rare, but also the most severe and tends to affect young and/or unhealthy individuals (Barriga, 1997). In this form of the disease, the worm burden is extremely high (> 10,000 worms) and the only sign of infection is the sudden death of the animal. Death often occurs within a week and is brought on by a severe anemia due to the large number of worms consuming the host blood (Dunn, 1978).

In the acute form of haemonchosis, the worm burden is moderate, 1,000-10,000 individuals, and all ages of animals are affected, regardless of current health status. The signs of infection are visible and include anemia, edema, lethargy, and wool loss. Anemia develops rapidly and the host mounts an erythropoietic response resulting in the production of red blood cells (Soulsby, 1982). Ewes often suffer from agalactia in which their ability to produce milk is lost and suckling lambs will often die due to malnutrition. Death may occur with acute haemonchosis, but may take several weeks to transpire (Dunn, 1978).

The third and final form of the disease is chronic haemonchosis. This is the most widespread of the three disease forms and often affects the entire flock or herd. Chronic haemonchosis is a result of a low worm burden, 100-1,000 individuals, and the most prominent clinical sign is the appearance of malnutrition (Soulsby, 1982). Anemia and edema are not usually present, and death is rare (Dunn, 1978). This type of haemonchosis is most often seen during dry periods when the pasture is in poor

condition and the host immune system is already being compromised by poor nutrition (Urquhart et al., 1987).

Early diagnosis of haemonchosis is essential for the treatment and survival of a flock or herd afflicted by this invasive parasite. Generally, a diagnosis for the acute and chronic forms of this disease is based on clinical signs as well as history. Fecal testing can also be performed to support the diagnosis. For hyperacute haemonchosis, the only way a diagnosis may be made is by performing a necropsy and looking for *H. contortus* adults in the abomasum of the deceased animal (Urquhart et al., 1987).

Many sheep do not develop an effective acquired immunity to *H. contortus* infections. However, some breeds, such as the Florida Native, St. Croix and Barbados Blackbelly, are less susceptible than others, such as the Rambouillet (Courtney et al., 1985). Amarante et al. (1999a, 1999b) crossed Florida Native sheep, which are small and resistant to *H. contortus* infection, and Rambouillet sheep, which are larger, faster growing, good wool producers and susceptible to *H. contortus* infections, to investigate if the offspring would possess the more desirable characteristics and show resistance to *H. contortus* infections. Upon challenge with the parasite, the F_1 generation did show a level of resistance. When the F_1 generation was crossed with each other, the resulting F_2 generation showed an even higher level of resistance (Amarante et al., 1999a, 1999b), which shows that crossing resistant breeds with susceptible ones could be valuable in developing a line of sheep with desirable production characteristics that is resistant to *H. contortus* infections.

Conflicting evidence questions the role of hemoglobin type in *H. contortus* resistance within certain breeds. In one study, Scottish Blackface and Finn Dorset sheep with type A hemoglobin possessed lower worm burdens and fecal egg counts and presented with less severe clinical signs than those with type B hemoglobin (Altaif and Dargie, 1978a, 1978b). However, many of the breeds that are considered to be highly resistant to *H. contortus* are predominantly type B while others, such as the Florida Native, are predominantly type A (Jilek and Bradley, 1969; Agar et al., 1972). Thus, the role of hemoglobin type is not clear in resistance of sheep to *H. contortus* infections.

To cope with gastrointestinal parasitic infections, sheep may undergo a phenomenon known as "self-cure" in order to alleviate the burden of *H. contortus* adults. Self-cure is a process in which the majority of adult parasites are expelled from the host when induced experimentally by a challenge dose of infective larvae or by the natural ingestion of a large number of infective larvae (Soulsby, 1982). As the larvae develop from the L_3 stage to the L_4 stage, an immediate-type hypersensitivity reaction develops to *H. contortus* antigens, leading to the expulsion of the adult worms. The host as well as the parasite each benefit from the self-cure process. The host is temporarily relieved of feeding adults and the damage that they cause, while the adult parasite population is replenished with a new generation (Urquhart et al., 1987).

Treatment of haemonchosis is accomplished by targeting the parasite itself. Today, this is accomplished through the use of anthelmintics, but over the past 200 years, several methods have been tried. Some of the earliest forms of treatment included arsenic and turpentine. These were highly toxic to the host, so new forms of treatment

were constantly being tested. One of the earliest and most significantly efficient forms of treatment was an oral dose of copper sulphate, which was widely used from the late 1800s to around 1940 (Gibson, 1975). When administered correctly, copper sulphate had very few toxic effects on the host and was effective against the adult worms but not the immature larval stages (Morgan, 1949). However, copper must be administered with caution since sheep are sensitive to chronic copper toxicity. Current evidence has shown that small doses (0.5-2 grams) of copper oxide wire particles given annually may be effective in controlling *H. contortus* infections in lambs without being toxic (Miller et al., 2005; Burke and Miller, 2006; Fleming et al., 2006).

In 1940, phenothiazine was introduced and replaced copper sulphate as the drug of choice. This drug proved to be highly effective for not only eliminating infections, but also for controlling outbreaks. Administration of phenothiazine was often through the use of a salt lick with the ideal concentration of a 1:10 phenothiazine to salt concentration. This proved to be a highly efficient dosing method with minimal toxic side effects (Gibson, 1975).

However, by the 1960s *H. contortus* began to show signs of resistance to phenothiazine, leading to the development of new anthelmintics. In 1961, the first to be introduced was a benzimidazole called Thiabendazole (Gibson, 1975). Although highly effective against both the adult and immature stages, *H. contortus* began to show signs of resistance to this anthelmintic after being in use for just a few years (Drudge et al., 1964; Soulsby, 1982). Several other anthelmintics followed, including additional

benzimidazoles, imidazothiazoles (levamisole), and macrocyclic lactones (ivermectin). These three classes of drugs are currently used to treat *H. contortus* infections.

Each class of anthelmintics possesses a distinct mode of action against parasites (Kohler, 2001). The target of benzimidazoles is the tubulin within the parasite intestinal cells, which forms into microtubules that are necessary for nutrient acquisition (Sangster and Dobson, 2002). Benzimidazoles bind to the β-tubulin component preventing it from forming microtubules within the intestinal cells of the helminth. This impairs the uptake of nutrients and inhibits the transportation of necessary digestive enzymes resulting in parasite death due to starvation (Kohler, 2001; Mansour, 2002). Imidazothiazoles, such as levamisole, are acetylcholine agonists that affect the nervous system of the parasite (Kohler, 2001). These drugs cause muscle contraction and paralysis in the helminth, resulting in the eventual expulsion of the parasite from the body (Craig, 1993; Mansour, 2002). Finally, macrocyclic lactones act on glutamate-gated chloride channels (GluCl). These drugs cause paralysis of the parasite neuromusculature, including the pharynx, preventing the worm from feeding (Kohler, 2001; Winterrowd et al., 2003). Benzimidazoles and macrocyclic lactones are effective against the adult and immature stages of the parasite, while the imidazothiazoles are effective against the adults and the later stages of immature larvae. These three classes of anthelmintics have proven to be successful in treating *H. contortus* infections.

Resistance to anthelmintics continues to be a growing concern in the treatment and control of *H. contortus* throughout the world. Anthelmintic resistance is defined as a genetically transmittable trait in which the sensitivity to a particular drug is lost in a

population of worms over time (Kohler, 2001). Through the use of anthelmintics, susceptible populations are being removed from the gene pool allowing the resistant populations to pass on their genes to successive generations. By 1994, resistance to the majority of the anthelmintics including the benzimidazoles, levamisole, and ivermectin was reported (Barriga, 1997). In addition, *H. contortus* populations are showing resistance to multiple anthelmintics (Sangster et al., 1999). Extensive use of drug treatments, whether proper or improper, and the ability of *H. contortus* to adapt to and overcome the deleterious effects of the drugs, have led to the development of drug resistance and, therefore, to the success of the parasite.

The key to controlling anthelmintic resistance in *H. contortus* is to understand the many mechanisms that may be involved, since each class of anthelmintics has a known different target. There are three main groups of mechanisms: those that change the binding sites of drugs, those that detoxify, and those that involve the active efflux of drugs by membrane transporters (Kerboeuf et al., 2003). The mechanism involved in resistance to each class of anthelmintic may be different or there may be a common one amongst all three classes.

In 1995, Kwa et al. suggested that a mutation in the β-tubulin is most likely the cause of resistance to the class of benzimidazoles. This mutation results in a single amino acid substitution from Phe200 to Tyr200 in the β-tubulin isotype 1 allele of resistant *H. contortus* strains. This mutation causes a decrease in the high affinity binding of the anthelmintic allowing for microtubule formation to occur in the presence

of the drug (Prichard, 1994). Without the inhibitory action of the anthelmintic, the parasite is capable of acquiring nutrients that are essential to its survival.

Resistance to levamisole in *H. contortus* is believed to be associated with an alteration in the nicotinic acetylcholine receptor found on the body muscles of nematodes. Responsible for the conduction of sodium, potassium and calcium through the muscle membranes, this receptor consists of five subunits (2α, 1β, 1γ and 1δ) arranged around a central ion-channel (Martin et al., 1997). Under normal conditions, the channel is closed, but in the presence of a ligand, such as levamisole, the channel may be opened. This opening of the channel allows ions to pass through, aiding in the muscle contraction and paralysis of the helminth (Martin and Robertson, 2000). Alterations in the acetylcholine receptor may lead to fewer receptors for levamisole to bind to or to a reduction in the receptor's sensitivity, which could prevent the channel from opening (Prichard, 1994). These alterations may also result in the shut down of the channel or in a blockage of the channel by the large levamisole molecule, interfering with the effectiveness of levamisole as an anthelmintic (Martin and Robertson, 2000).

The mechanisms involved with macrocyclic lactone resistance are not fully understood (Kohler, 2001). Blackhall et al. (1998a) correlated the selection of an altered GluCl gene with resistance to ivermectin. Glutamate-gated chloride channels are believed to be similar in structure to acetylcholine receptors in that they consist of five subunits (α and β) that come together to form a central ion-channel (Martin et al., 1997, 1998). The α-subunits contain the glutamate binding site and the β-subunits contain the ivermectin binding sites (Martin et al., 1997). The selection of a single allele of the

α-subunit was found in increased frequency in resistant strains of *H. contortus* (Blackhall et al., 1998a). The mutation that results from this allelic selection may interfere with the conformational changes induced by the drug binding and may not actually inhibit the binding itself (Kerboeuf et al., 2003). The γ-aminobutyric acid (GABA) receptor gene has also been suggested as a mechanism for macrocyclic lactone resistance. Its function is similar to GluCl and so an alteration in its gene may also contribute to resistance (Blackhall et al., 2003).

The mechanism that is primarily considered to be involved in resistance to macrocyclic lactones is the detoxification process of P-glycoproteins. P-glycoproteins (Pgp) are efflux transporters that belong to the ATP binding cassette (ABC) superfamily which actively transport compounds, including drugs, across membranes (Sangster and Dobson, 2002). The hydrolysis of ATP is required for the efflux of xenobiotics (chemicals foreign to the organism) by Pgp to occur (Sharom, 1997). P-glycoproteins are predominately confined to the digestive tract and are highly expressed on the membranes of intestinal and pharyngeal cells (Smith and Prichard, 2002). The primary function of Pgp is to protect the organism by actively pumping toxic substances out of its cells (Sangster, 1994; Geick et al., 2001; Thompson and Geary, 2002).

P-glycoproteins are highly conserved transmembrane proteins (Sangster and Dobson, 2002). They are made up of two homologous halves, each with six transmembrane domains (TM) and one nucleotide binding domain (NBD) (Sangster, 1994; Sharom, 1997; Ambudker et al., 1999). The highly conserved NBDs are separated by an internucleotide binding domain (IBD) which allows both halves to interact and

work together as a single transporter (Ambudker et al., 1999; Sangster et al., 1999). Anthelmintic binding occurs within the TM while ATP binding and hydrolysis occur in the NBD (Sangster, 1994; Sharom, 1997; Ambudker et al., 1999; Sangster et al., 1999). The normal function of Pgp in nematodes is not fully understood, but due to its capability to bind a wide range of substrates including anthelmintics, the function of Pgp may be to protect the organism from toxic substances (Sharom, 1997; Ambudker et al., 1999).

P-glycoproteins have been identified in *H. contortus* and the full cDNA sequence has been obtained (Xu et al., 1998). At least 7 genes are known to be involved in encoding Pgp in *H. contortus*, allowing for numerous isoforms (Kerboeuf et al., 2003). In addition to the full cDNA sequence, numerous internucleotide binding domains (IBD) within Pgp have also been sequenced (Sangster, 1994; Sangster et al., 1999). Each IBD is sequentially different, and one IBD in particular, correlates with resistance to the macrocyclic lactone avermectin/milbemycin (Sangster et al., 1999). The combinations of the different genes and their variable IBDs allow for considerable variation in the Pgp of *H. contortus*. This may contribute to the binding of a wide variety of substrates and possibly to the development of anthelmintic resistance (Xu et al., 1998; Sangster et al., 1999).

In vertebrates, Pgp is encoded for by the multidrug resistance gene, MDR1. This gene is activated by a family of nuclear receptors that includes the human SXR (steroid and xenobiotic receptor) and its animal homolog PXR (pregnane X receptor) (Xie et al., 2000; Geick et al., 2001; Synold et al., 2001). These nuclear receptors are found in the

intestine and enhance the removal of xenobiotics by Pgp. This is accomplished through the regulation of the transcription of the cytochrome P450 (CYP) gene product CYP3A, which is involved in the oxidative metabolism of a variety of steroid hormones and xenobiotics (Xie et al., 2000; Synold et al., 2001, Ding and Staudinger, 2005). The receptors, SXR/PXR, must form a heterodimer with RXR (retinoic acid receptor) which enables the molecule to bind to specific DNA sequences, including those of CYP3A (Kliewer et al., 1998; Masuyama et al., 2001). The activation of SXR/PXR by a variety of agents may contribute to pharmaceutical resistance as well as regulate multidrug resistance (Synold et al., 2001).

Currently, a SXR/PXR homolog has not been identified in nematodes. The complete genome of *Caenorhabditis elegans*, a free-living nematode, has been sequenced and the RXR, or a homolog, is not present or has not yet been determined (Enmark and Gustafsson, 2000; verified by a protein-protein BLAST). However, numerous CYP genes have been identified in nematodes. Gotoh (1998) determined that *C. elegans* possesses at least 60 potentially active CYP genes. These genes are closely related to the CYP genes in vertebrates and may function in the catabolism of xenobiotics.

The activity of CYP in *H. contortus* may depend on the environment in which the parasite lives. Cytochrome P450 has the ability to catalyze substrates as a monooxygenase, which requires molecular oxygen, or as a peroxygenase, which does not. In 1997, Kotze found that the monooxygenase catalysis of certain substrates by CYP was readily detectable in the free-living stages of *H. contortus*, but considerably

lower or absent in the adult stages and attributed this to the level of oxygen present. In oxygen-poor environments, CYP may function as a peroxygenase, thereby utilizing hydroperoxide to catalyze substrate oxidations without requiring molecular oxygen (Kotze, 1999). The role of CYP in metabolizing xenobiotics in *H. contortus* is not yet fully understood, but may play a role in anthelmintic resistance.

Since *H. contortus* has developed resistance to each of the classes of anthelmintics, a common mechanism may be involved. The mechanism believed to be associated with anthelmintic resistance in *H. contortus* is the overexpression of Pgp. Benzimidazoles, levamisole and ivermectin possess characteristics that are common to Pgp substrates. These include a planar shape, at least one ring structure, hydrophobic properties and they are amphiphilic (a molecule possessing a polar, water-soluble group attached to a non-polar, water-insoluble hydrocarbon chain) (Ford and Hait, 1990; Ambudkar et al., 1999).

Both benzimidazole-resistant and ivermectin-resistant strains of *H. contortus* have been found to possess Pgp alleles in higher frequency than susceptible strains. For benzimidazoles, Pgp may modulate drug concentration at the target site (Kerboeuf et al., 2003). In humans, it has been determined that benzimidazoles bind to Pgp in multidrug-resistant (MDR) lymphoma cells (Nare et al., 1994). A relationship between Pgp and benzimidazole resistance was indirectly demonstrated through the use of the Pgp inhibitor verapamil (Beugnet et al., 1997). Verapamil is a calcium channel blocker, which actively inhibits the Pgp drug-binding domain. When given in conjunction with an anthelmintic, the efflux of the drug is reduced, resulting in its increased efficacy

(Molento and Prichard, 1999). The experiments conducted by Beugnet et al. (1997) showed that, in the presence of verapamil, the toxicity of the drug increased and that benzimidazole resistance could be partially reversed.

The role of Pgp in macrocyclic lactone resistance, especially ivermectin, is better understood. Ivermectin has been described as a possible substrate for Pgp in nematodes (Xu et al., 1998; Blackhall et al., 1998b). In 1998, Xu et al. found higher levels of Pgp in ivermectin-resistant *H. contortus* populations than in susceptible populations. They also found alterations in the structure and/or transcription of Pgp that resulted in its overexpression, which may modulate drug concentration at the target site. When verapamil was given to ivermectin-resistant strains of *H. contortus,* the efficacy of ivermectin was increased, similar to that seen in the benzimidazole resistant strains (Xu et al., 1998). The role of Pgp in resistance to levamisole is not known, however, levamisole may act as a substrate for Pgp much like ivermectin (Kerboeuf et al., 2003) Based on this information, Pgp transport may be an important mechanism in anthelmintic resistance.

Since Pgp appears to play a role in resistance to different drugs, the occurrence of multidrug-resistance (MDR) may be explained by this one mechanism. The overexpression of Pgp has been associated with MDR in tumor cells in humans and has been suggested as the mechanism of resistance in nematodes (Kerboeuf et al., 2002). Through the use of MDR-reversing agents, such as verapamil, reversal of resistance can be accomplished (Molento and Prichard, 1999).

There are several methods available to determine the presence of anthelmintic resistance in a population of *H. contortus*. These methods include in vivo as well as in vitro tests and there are advantages as well as disadvantages associated with each one. In vivo tests are time consuming, expensive and not very reliable. In vitro tests are more technically demanding, but are more accurate and may be used to detect resistance to multiple anthelmintics at one time (Craven et al., 1999).

The most commonly used in vivo method is the fecal egg count reduction test (FECRT) and is considered to be the gold standard. The FECRT utilizes the modified McMaster technique to compare egg counts before and after anthelmintic treatment to give an estimate of anthelmintic efficacy. This is a simple test and may be used to detect resistance to all classes of anthelmintics (Coles et al., 1992; Craven et al., 1999; Fleming et al., 2006). However, the FECRT may give false indications of levamisole resistance due to the development of immature larvae which are not affected by treatment (Grimshaw et al., 1996; Taylor et al., 2002; Coles et al., 2006). The results of the FECRT are subject to interpretation and the parasitic species present can not be directly determined; therefore, the sample must also be cultured in order to identify the species present (Vizard and Wallace, 1987; Coles et al., 2006).

The egg hatch test (EHT), the larval development assay (LDA), and the recently developed molecular testing are the prominent methods used for in vitro testing. The EHT is used for testing resistance to the benzimidazoles. First described by Le Jambre (1976), eggs are hatched in serial dilutions of anthelmintic and the level of resistance is determined. There are several factors which can influence the outcome of this test

including the age of the feces and the development level of the eggs at the start of testing (Taylor et al., 2002; Coles et al., 2006). One advantage to the EHT is that the larvae which hatch can be identified to determine the parasitic species which is resistant. However, similar to the FECRT, the results of the EHT are subject to interpretation (Taylor et al., 2002).

Larval development assays (LDA) are capable of determining the level of resistance to all three classes of anthelmintics simultaneously. One such test is the DrenchRite® Assay (CSIRO) in which eggs are loaded onto a 96-well microtiter plate containing agar with serial concentrations of each drug. Resistance is determined by analyzing the level of larval development after a period of incubation (Tandon and Kaplan, 2004). In 2001, Terrill et al. showed that results obtained from the DrenchRite® assay were consistent with FECRT, which is considered to be the gold standard. Larvae can be identified directly from the plate in order to determine the resistant species present (Coles et al., 2006).

In recent years, molecular diagnosis of anthelmintic resistance has become a focus of research. Several studies have shown polymerase chain reactions (PCR) to be highly accurate and sensitive in determining resistance. However, this type of testing is very expensive and must be conducted on an adequate sample size to provide significant results, which is not always possible. The development of a molecular test, which is capable of determining the level of resistance in a parasitic population from a pooled DNA sample, will be fundamental to the advancement of molecular testing of anthelmintic resistance (Coles et al., 2006; von Samson-Himmelstjerna, 2006).

Prevention of *H. contortus* infections is difficult. Since there are no vaccines available, the best preventative method is good pasture management. If a flock or herd has been diagnosed with *H. contortus* infections, then in addition to treatment with anthelmintics, the flock or herd should be moved to a non-infested pasture (Urquhart et al., 1987). The infested pasture should not be used until there is no longer evidence of the presence of *H. contortus,* which is impossible. Also, limiting the number of individuals and alternating the species grazing on a pasture may be helpful in controlling infections (Dunn, 1978). It is not recommended that the pasture be treated, since this will only increase the incidence of resistance by eliminating susceptible worms leaving behind only resistant worms to infect hosts and treatment of the pasture is difficult (Barriga, 1997).

Haemonchus contortus is a very problematic parasite. Found worldwide, this parasitic nematode can inflict a significant amount of damage to a population of ruminants in a short period of time. High parasitism levels are possible, due to the proficiency of the females as egg layers, the efficiency of the life cycle, and the high survivability of the larvae. Haemonchosis can often lead to the death of a significant number of individuals within a flock/herd if not properly diagnosed and treated. Many classes of anthelmintics are used as treatments with varying modes of action. Benzimidazoles, imidazothiazoles and macrocyclic lactones are the most commonly used due to their effectiveness. However, resistance to the majority of anthelmintics used today is developing rapidly in *H. contortus.* Several studies have been conducted

to determine the mechanisms responsible for drug resistance. Hopefully, these studies will aid in the development of new methods for the control of this devastating parasite.

The objectives of this study were to determine the anthelmintic resistance/susceptibility status of *H. contortus* strains using a larval development assay and to compare the levels of transcription of the transporter protein, P-glycoprotein, in these strains before and after exposure to anthelmintics. Our hypothesis was that P-glycoprotein will be expressed in higher levels upon exposure to anthelmintics and that higher levels will be seen in anthelmintic resistant populations of *H. contortus* compared to susceptible populations.

2. MATERIALS AND METHODS

2.1 Parasite strains

A total of seven strains of *H. contortus* were evaluated in this study (Table 1). Four strains suspected to be anthelmintic susceptible were obtained from mixed sheep/goat farms in Texas. These included *H. contortus* – Eldorado Sheep (Hc-ES), *H. contortus* – Eldorado Goat (Hc-EG), *H. contortus* – Ozona Sheep (Hc-OS) and *H. contortus* – Ozona Goat (Hc-OG). For all of these strains, L_3 larvae were inoculated into helminth-free sheep and goats and housed in a controlled environment at the Texas A&M University Research Farm.

Three of the seven strains were suspected to be anthelmintic resistant. Two of these were obtained from animals on the Texas A&M University Research Farm. The first, Hc-H992, was obtained approximately ten years ago and has been maintained in a controlled environment without exposure to anthelmintics. The second, Hc-RFR, was recently acquired from a mixed population of sheep and goats on the Research Farm, which had been continuously exposed to anthelmintics. For both strains, L_3 larvae were inoculated into helminth-free sheep and goats and housed in a controlled environment. The third suspected resistant strain (Hc-GRF) was submitted by a zoo in Florida to the Texas A&M University Diagnostic Parasitology Laboratory for diagnostic evaluation. The sample was from a giraffe housed at the zoo that had previously been diagnosed and treated for *H. contortus* and was currently not responding to anthelmintic treatment. The strain was inoculated into a helminth-free sheep housed at the Research Farm and housed in a controlled environment.

Table 1
Haemonchus contortus strains

Strain	Host	Geographic Origin	Suspected Anthelmintic Status
Hc-ES	Sheep	Eldorado, Texas	Susceptible
Hc-EG	Goat	Eldorado, Texas	Susceptible
Hc-OS	Sheep	Ozona, Texas	Susceptible
Hc-OG	Goat	Ozona, Texas	Susceptible
Hc-RFR	Sheep/Goat	College Station, Texas	Resistant
Hc-H992	Sheep/Goat	College Station, Texas	Resistant
Hc-GRF	Giraffe	Loxahatchee, Florida	Resistant

2.2 Modified McMaster test

For each strain of *H. contortus*, feces were collected directly from the rectum (except for the Hc-GRF strain, which was collected from the ground). A modified McMaster test was conducted on the feces to determine the eggs per gram (EPG) using a McMaster slide. This provided an estimate of the quantity of eggs present in the fecal sample to determine whether sufficient eggs were present for conducting a larval development assay (DrenchRite®, Horizon Technology Pty Limited, Roseville, NSW, Australia). A fecal solution was made in a vial by combining 28 ml of saturated sodium chloride (specific gravity 1.2) with 2 grams of feces. The feces were carefully broken apart and the solution was mixed by gentle inversion. Immediately following mixing, a sample of the fecal solution was pulled from the center of the vial and the two chambers

of the McMaster slide were filled with the solution. At 100X magnification, the number of eggs that fell within the grid of each chamber was counted and EPG was calculated as follows:

$$\frac{\text{Grid 1 + Grid 2}}{2} \quad X \quad 100 \quad = \quad EPG$$

The EPG was then multiplied by the weight of the remaining feces to provide an estimate of the number of eggs in the fecal sample.

2.3 Copro culture, Baermann technique, and larval identification

Since worm identification cannot be determined based on the trichostrongyle egg, copro cultures of feces were performed to allow for the development of L_3 larvae, which can be identified to genus. For each strain, approximately 20 grams of feces were placed in the center of a piece of cheesecloth and the sides were tied together using cotton string to form a fecal packet. The bottom of a culture jar was filled with approximately 25-30 ml of tap water and the fecal sample was suspended 5-6 cm above the surface of the water by tightening the lid on the string (Fig. 1A). The jar was incubated at room temperature for 7 days to allow parasite development to the L_3 larval stage.

Upon completion of the incubation, the fecal sample was transferred to a Baermann apparatus for larval collection. The gauze containing the feces was placed into a funnel attached to a 15 ml conical tube by rubber tubing and the entire set-up was set in a graduated cylinder which served as a funnel stand (Fig. 1B). Warm tap water

was added to the funnel until the fecal sample was adequately covered. The larvae were allowed to emerge from the feces and collect in the bottom of the conical tube for approximately 2-3 h.

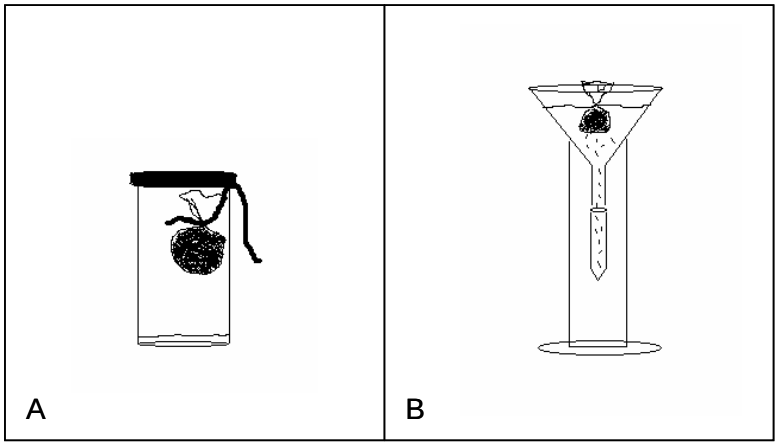

Fig. 1. (A) Drawing of a copro culture set-up. (B) Drawing of a Baermann apparatus.

Larvae were transferred to a glass slide using a transfer pipette, cover-slipped, and immobilized with a dilute solution of Lugol's iodine for identification. The larvae were examined under 400X and 1000X magnification and identification was based on morphological characteristics using a L_3 identification key (from The Manual of Veterinary Parasitological Laboratory Techniques modified by C.G. Wade, Texas A&M University, Appendix A). The first step was to determine if the esophagus was rhabditiform or non-rhabditiform. For *H. contortus*, the esophagus is non-rhabditiform. Next, it was determined 1) whether there was a sheath present, 2) if the esophagus was ¼

or ½ the length of the body and 3) if the tail was notched or tapered. For *H. contortus*, there is a sheath present, the esophagus is ¼ the length of the body and the tail is tapered. The length of the tail of the sheath was then determined to be either short (< 50 µm), medium (> 50 µm < 100 µm), or long (> 100 µm). Finally, the anterior of the larvae was examined for the presence of refractive bodies or a bright transverse band between the buccal cavity and the esophagus. The tail of the sheath for *H. contortus* larvae is medium length, and is often kinked, and the larvae do not possess refractive bodies or a bright transverse band at the anterior end. The remaining larvae were dispensed in aliquots of 50 larvae, washed extensively, and stored at -80 °C for later use in molecular analysis.

2.4 Egg isolation

A modified protocol of the DrenchRite® User Manual was followed to isolate the eggs from the feces for each of the strains tested. The weight of the feces was determined in grams and combined with 1 ml of tap water per gram of feces in a plastic beaker and allowed to soak for 30 min. The feces were broken up using a wooden tongue depressor and at least 2 ml of tap water per gram of feces was added to make a fecal slurry.

The fecal slurry was first washed with tap water and passed through a 250 µm sieve. The debris retained on the sieve was saved until it was determined that an adequate quantity of eggs had been isolated. The filtrate was collected and allowed to settle for 30 min, and then the top ⅔ was decanted and discarded. The resulting

sediment was similarly washed through a 180 µm sieve followed by a 75 µm sieve with tap water and the filtrate was collected for each wash and allowed to settle for 30 min. The top ⅔ of the filtrate was decanted and discarded as before and the debris on the sieves was saved for each step. Depending on the condition of the feces, additional washings through the 75 µm sieve were conducted to remove as much debris as possible. The final sediment was filtered through a 37 µm sieve and washed with tap water. The material containing the eggs that was collected on the 37 µm sieve was back washed with a minimal volume of tap water into a clean beaker and allowed to settle for 30 min. Using a 30 cc syringe, excess water was carefully removed from the sediment.

A sugar gradient was used to separate the eggs from any residual debris. The gradient was prepared in a 50 ml conical tube using 10%, 25% and 40% sugar solutions. First, 10 ml of the 10% sugar solution (yellow solution in DrenchRite® protocol) was added to the tube using a large bore Luerlock needle and a 20 cc syringe. Next, 10 ml of the 25% sugar solution (blue) was added beneath the 10% solution. Then, 15 ml of the 40% sugar solution (red) was added beneath the 25% sugar solution. Finally, 10-15 ml of the egg slurry was carefully layered on top of the sugar gradient. The tube was placed in a bench top centrifuge and centrifuged for 7 min at 2450 X *g*. The eggs were collected from the sugar gradient using a 1 ml transfer pipette at the yellow/blue interface where a distinct white band could be seen in a green area of the gradient. The eggs were transferred to a plastic cup, and then washed on a 37 µm sieve with distilled water to remove the sugar. The eggs were then back washed into a plastic cup with

distilled water, transferred to several 15 ml conical tubes, and placed in 4 °C overnight to allow the eggs to settle.

Once the eggs had settled to the bottom, excess water was removed from each of the 15 ml conical tubes and the eggs were pooled into a single tube. The eggs were allowed to settle for 30 minutes and excess water was removed until the final volume was 2 ml. The eggs were resuspended by vortexing and 20 µl was transferred to a glass slide and cover-slipped. An egg count was conducted at 100X magnification to determine the concentration of eggs isolated. The egg concentration was then adjusted to ~ 50 eggs per 20 µl and Fungizone (provided in kit) was added per the DrenchRite® protocol and the solution was mixed well.

2.5 DrenchRite® assay

The larval development assay, DrenchRite® (DR), is an in vitro assay to determine anthelmintic resistance in gastrointestinal parasitic nematodes of ruminants The DR assay consists of wells in a 96-well microtiter plate containing agar with increasing concentrations of benzimidazole (BZ), levamisole (LEV), avermectin/milbemycin (AVM) or benzimidazole/levamisole in combination (BZ/LEV) across the rows (Fig. 2). There are duplicate wells for each anthelmintic concentration, except for the avermectin/milbemycin group, and the plate is color-coded: clear for control wells (no anthelmintic), green for susceptible, yellow for weak resistant, and pink for resistant.

Each DR plate was removed from the aluminum pouch and examined for dehydrated wells and varying agar amounts. For any dehydrated well, 10 µl of distilled water was added to the well. Based on observation, the perimeter wells were often dehydrated or would become dehydrated within the first 24 hours of the assay, so 10 µl of distilled water was automatically added to these wells for each plate.

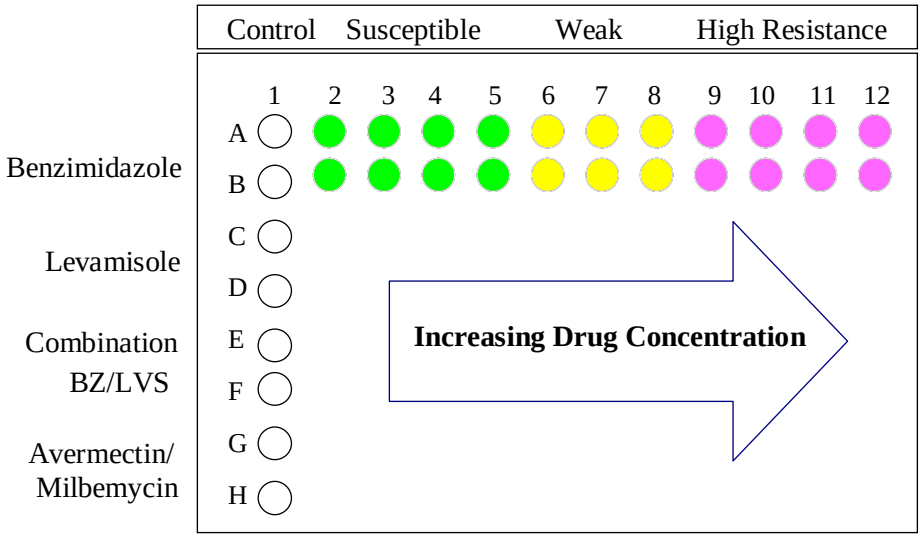

Fig. 2. Diagram of the DrenchRite® assay plate. (Adapted from the DrenchRite® Manual).

For each strain, 20 µl of the egg suspension (~50 eggs) described above was added to each well of the plate. The suspension was vortexed after dispensing into every 4th well to keep the mixture homogeneous. The eggs in each well were counted under 10-45X magnification on a dissecting scope and the count was recorded on a log sheet (Appendix B). The plate was placed in a humidified 25 °C incubator. Any remaining

egg solution was transferred to a 250 ml canted neck flask and placed in the incubator alongside the plate. Distilled water was added to the flask until the bottom was adequately covered.

The plate was checked for dehydration and larval development approximately 20 h after adding the eggs. If any of the wells were dehydrated, 10 µl of distilled water was added to the well. After 24-48 h of incubation, 20 µl of growth medium (included in kit) was added to each well of the plate and 1-2 ml was added to the flask. The plate was checked daily for dehydration and to monitor larval development. After 168 h (approximately 7 d) of incubation, the plate was ready for interpretation.

2.6 DrenchRite® larval counts and collection

Each well of the assay plate was inspected and the L_1/L_2 larvae and L_3 larvae were counted under 10-45X magnification on a dissecting scope. The DrenchRite® protocol recommended adding a dilute solution of Lugol's iodine to each well to kill the larvae. However, for the purpose of this study, the larvae were not killed so the larvae could be collected and used for molecular analysis. For each well, the L_1/L_2 larvae were counted in the well and the number was recorded on the log sheet (Appendix B).

Starting with the control wells (A1-H1), the L_3 larvae from each well were transferred to a 3-well depression slide (one well per depression) using a 100 µl pipette with a wide-bore tip. The L_3 were counted and the surviving L_3 count was recorded separate from the count of any L_3 larvae that had died. The surviving L_3 were then collected from the slide using a 100 µl pipette with a fine tip. Aliquots of 50 larvae were

transferred to 2 ml tubes starting with the first well (A1) and pulling from the next well (B1) and so on down the control wells to complete the 50 until all surviving L_3 larvae had been transferred to a tube.

The L_3 larvae for the wells containing anthelmintic (wells 2-12) were treated in much the same way. Starting with the BZ larvae in wells A2 and B2, the larvae were counted, the number was recorded on the log sheet, and the surviving larvae were collected. If there were not 50 larvae between wells A2 and B2, then larvae from well A3 were added, then B3 and so on until there were 50 larvae in the tube. The larvae were pooled according to the groupings in the plate: wells 2-5 (susceptible), wells 6-8 (weak resistant) and wells 9-10 (resistant). If there were not 50 surviving larvae in a group, then the number in the tube was recorded and the next group started anew. Once all the BZ larvae were counted and collected, the LEV, BZ/LEV combination and AVM larvae were counted and collected in the same manner.

After all of the L_3 larvae were collected from the plate, then the tubes were labeled and the larvae were washed. Dulbecco's phosphate buffered saline (PBS) with 500 U/ml penicillin, 500 µg/ml streptomycin, 1.25 µg/ml amphotericin B (Antibiotic/Antimycotic, Invitrogen, Carlsbad, California, USA) (PBS-AB/AM) was prepared and 500 µl was added to each tube of larvae and mixed. The tubes were placed on ice for a few minutes to allow the active larvae to become immobilized and collect in the bottom of the tube so that the PBS-AB/AM could be removed without loss of larvae. The PBS-AB/AM was transferred to a 3-well depression slide and examined under 10-45X magnification to ensure that no larvae had been removed. Any larvae that had

been removed were then returned to the tube using a 100 µl pipette. This was repeated with a second wash with PBS-AB/AM, followed by a third wash with PBS without antibiotics. After the final wash, as much PBS as possible was removed without losing any larvae. The tubes were centrifuged briefly to bring the contents to the bottom, flash frozen in liquid nitrogen, and stored in -80 °C until RNA isolation.

2.7 Determination of resistance

The level of resistance to each anthelmintic in the DR assay was determined for each strain tested based on the counts obtained above (Appendix B). The percentage of L_1/L_2 larvae in each well was calculated as follows:

Controls, BZ (wells 2 – 4), LEV, AVM and BZ/LEV:

$$\frac{\text{\# of } L_1/L_2 \text{ larvae}}{\text{Total \# of larvae}} \times 100 = L_1/L_2$$

BZ (wells 5 – 12):

$$\frac{\text{\# of } L_1/L_2 \text{ larvae + \# of unhatched eggs}}{\text{Total \# of eggs}} \times 100 = L_1/L_2$$

The percentage of L_3 larvae for all wells was calculated as follows:

$$\frac{\text{Total \# of } L_3 \text{ larvae}}{\text{Total \# of larvae}} \times 100 = L_3$$

The critical well, in which 50% of larval development to the L_3 stage was blocked, was estimated to the nearest half-well.

2.8 RNA isolation

Hc-OS, Hc-OG, Hc-EG, Hc-RFR, Hc-H992 and Hc-GRF were selected for molecular analysis based on the DrenchRite® assay results. For each strain, the anthelmintic exposure status of the tubes of larvae collected from the DR plate was designated as indicated in Figure 3 and several tubes containing 50 larvae each were selected for molecular analysis as shown in Table 2.

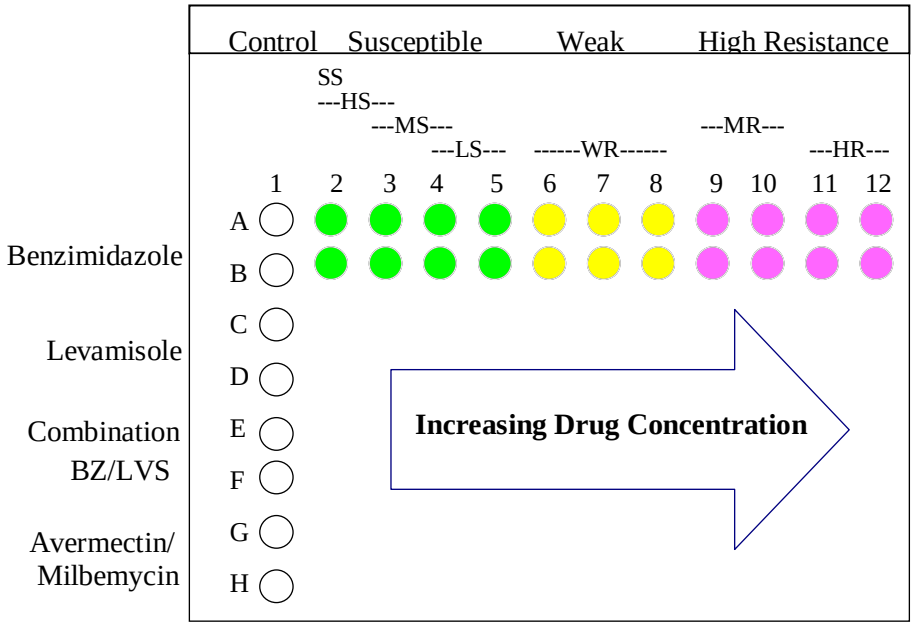

Fig. 3. The anthelmintic exposure designations for the tubes of larvae based upon the DrenchRite® assay plate. (SS = super susceptible (well 2), HS = highly susceptible (wells 2 and 3), MS = moderately susceptible (wells 3 and 4), LS = low susceptible (wells 4 and 5), WR = weakly resistant (wells 6, 7 and 8), MR = moderately resistant (wells 9 and 10), HR = highly resistant (wells 11 and 12).

Total RNA was isolated from the larvae samples selected for each strain of *H. contortus* using the TõTALLY RNA™ kit (Ambion, Austin, Texas, USA). For each tube, the larvae were ground to a powder under liquid nitrogen using a plastic pestle powered by a cordless motor (Kontes Glass Company, Vineland, New Jersey, USA). Denaturation Solution (200 µl) was added to the tube and the pestle was rinsed into the tube with an additional 200 µl of Denaturation Solution. The volume of the resulting lysate was measured and this volume was referred to as the Starting Volume. The RNA was then isolated following the Ambion protocol. The final RNA pellet was resuspended in 10 µl of DEPC Water/0.1mM EDTA (Ambion), placed in a 55 °C water bath for 15 min, and then stored at -80 °C.

2.9 cDNA synthesis

Reverse transcription to generate 3' cDNA was accomplished from the larvae total RNA preparations (above) using the SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, California, USA). Similarly, 3'-RACE-Ready cDNA was synthesized from total RNA isolated from adult *H. contortus* for use as a positive control. Following the kit protocol, 3 µl total RNA (approximately 125-225 ng) was used for each sample, the reactions were mixed gently by stirring after each step, and a hot-lid thermal cycler programmed to the appropriate temperatures was used for all incubations. In the final step, the reactions were diluted with 20 µl of Tricine-EDTA Buffer and incubated at 72 °C for 7 min. Once the reactions were complete, the first-strand cDNA was transferred to a 0.5 ml microcentrifuge tube and stored at -20 °C.

Table 2
Tubes selected for molecular analysis of P-glycoprotein transcription

	Hc-EG	**Hc-OS**	**Hc-OG**	**Hc-RFR**	**Hc-H992**	**Hc-GRF**	**TOTAL**
Controls	3	3	3	3	3	3	18
BZ							
SS						1	1
HS	1	2	2	1	1		7
WR	1		1	2	4		8
HR				1	1	1	3
LEV							
SS						1	1
HS		2	2		1		5
MS	1	2	2	2	2		9
HR						1	1
AVM							
HS	1	2	2	1	1	1	8
WR	1			1	1	1	4
TOTAL	8	11	12	11	14	9	65

The cDNA concentration was determined for each sample using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, Delaware, USA). After all of the concentrations had been measured, dilutions of the cDNA were made to obtain a concentration of approximately 150 ng/µl, which had been previously

determined to be an appropriate concentration for molecular analysis. The concentration of the dilutions was then confirmed by NanoDrop spectrophotometry and the exact volume of cDNA suspension that would give 150 ng was calculated for molecular analysis (Appendix C).

2.10 P-glycoprotein molecular analysis

The cDNA samples for each strain were analyzed by quantitative polymerase chain reactions (PCR) to determine the level of Pgp transcription. To amplify the entire gene, primers Pgp003-20F and PgpAF003-HcR were designed from a complete *H. contortus* Pgp gene sequence in GenBank (accession no**.** AF003908) (Table 3). Primers Hc18S-620F and Hc18S-1010R were also designed from the *H. contortus* 18S ribosomal RNA (rRNA) gene (GenBank accession no. L04153) for use as a housekeeping gene internal standard (Table 3). Due to the different optimal conditions, reactions for the Pgp and 18S PCR were set up separately for each strain, and included a negative (sterile water) and positive control (cDNA from adult worms). The PCR reactions were performed according to manufacturer's instructions (Advantage 2 PCR Enzyme System, Clontech, Mountain View, California, USA), except using 150 ng cDNA in a 12.5 µl volume. The Pgp PCR cycling parameters were 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 3 min, followed by a cycle of 72 °C for 10 min and then held at 4 °C. The 18S PCR cycling parameters were 30 cycles of 96 °C for 10 s and 72 °C for 1 min, followed by a cycle of 72 °C for 10 min and then held at 4 °C. The Pgp

Table 3
Primers for P-glycoprotein transcription analysis

Primer	Sequence (5´ to 3´)	T_A	Expected length
Pgp003-20F	AGAGATCGTTCTCAAGCTGGT	60 °C	3852 bp
PgpAF003-HcR	TCATTGTGATTCAACGAGTCGT		
Pgp003-3250F	ATGGCGTTGTTGGAACGGTTT	56 °C	141 bp
Pgp003-3400R	GGTACAGTCGAACAGCGTTGGTTCC		
Hc18S-620F	GAGTTACATGCAGTGATTCGCCTTTGGCGTTAATCGCTGTTG	72 °C	423 bp
Hc18S-1010R	GCTCCTCGACAAGGCAACTATACCCCATCGGAT		

and 18S PCR products were co-loaded and visualized on a 1% agarose gel, stained with ethidium bromide, and viewed under UV transillumination. The samples for each individual strain were amplified and evaluated under identical conditions.

Amplification of a smaller fragment of Pgp was also evaluated for some of the experimental samples. The primers Pgp003-3250F and Pgp003-3400R were designed from the *H. contortus* Pgp gene sequence in GenBank (accession no. AF003908) (Table 3). Reactions were set up as for the larger amplicon using 150ng per reaction. The PCR cycling parameters were 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, followed by a cycle of 72 °C for 10 min and then held at 4 °C. The products were

visualized on a 1% agarose gel, stained with ethidium bromide, and viewed under UV transillumination.

2.11 Molecular characterization of Hc-GRF strain

The 18S rRNA gene and the internal transcribed spacer 1 (ITS 1) and ITS 2 regions were analyzed from Hc-H992 and the *H. contortus* strain obtained from the giraffe (Hc-GRF).

2.11.1 Genomic DNA extraction

Genomic DNA (gDNA) was extracted from Hc-H992 and Hc-GRF strain L_3 larvae using a standard phenol-chloroform extraction method facilitated by the use of the Phase-Lock Gel tubes (Eppendorf Scientific, Inc., Westbury, New York, USA). The larvae were ground to a powder as described above. A 100 µl volume of PBS was added to the sample and the pestle was rinsed with an additional 50 µl of PBS. An equal volume of lysis buffer (10 mM Tris-chloride (pH 8.0), 0.1 M EDTA (pH 8.0), 0.5% SDS) was added to the tube and the lysate was transferred to a Light Phase-Lock Gel tube. RNAse A was added to the lysate at a final concentration of 50 µg/ml and the tube was incubated in a 37 °C water bath for 1 h. Then, Proteinase K was added to a final concentration of 100 µg/ml concentration and incubated in a 50 °C water bath for 3 h with occasional swirling. The sample was cooled to room temperature.

A volume of Tris-equilibrated phenol equal to the sample volume was added to the tube and mixed gently on a rocker for 30 min then centrifuged at 10,000 X *g* for

8 min. The volume of the top aqueous phase was estimated and an equal volume of Tris-equilibrated phenol was added. The tube was mixed gently on a rocker for 10 min then centrifuged at 10,000 X *g* for 8 min. The top aqueous phase was measured and transferred to a Heavy Phase-Lock Gel tube. An equal volume of 50:50 chloroform:phenol was added to the sample and mixed for 5 min. The tube was centrifuged at 10,000 X *g* for 8 min. The volume of the top aqueous phase was estimated and an equal volume of chloroform/iso-amyl alcohol was added. The sample was mixed for 5 min, and then centrifuged at 10,000 X *g* for 8 min. The top aqueous phase was transferred to a 2 ml RNAse/DNAse free tube, the volume was measured and sodium acetate to a final concentration of 0.3 M was added. The tube was mixed gently and 3 volumes of cold absolute ethyl alcohol (EtOH) were pipetted down the side of the tube and mixed. The sample was placed in -80 °C to precipitate overnight.

The sample was centrifuged for 30 min at 12,000 X g at 4 °C. The EtOH was carefully removed from the pellet and 500 µl cold 70% EtOH was added, and then centrifuged for 10 minutes at 12,000 X *g* at 4 °C. The EtOH was removed, the pellet was allowed to dry, and 20-50 µl of Tricine-EDTA Buffer was added directly to the pellet for resuspension. To ensure that the pellet was fully resuspended, the tube was placed in a 50 °C water bath for 1 h. The concentration of the gDNA was measured on a NanoDrop Spectrophotometer.

2.11.2 18S rRNA standard PCR

Polymerase chain reactions were conducted for the 18S small subunit rRNA gene (18S) and the ITS region of the Hc-GRF and Hc-H992 gDNA. For the 18S PCR, primers AN and B were designed from a conserved region of the gene (Sogin, 1990; Schoelkopf et al., 2005) and used for amplification (Advantage 2 PCR Enzyme System, Clontech, Mountain View, California, USA) (Table 4). PCR reactions contained 10 ng Hc-GRF gDNA, 30 ng Hc-H992 gDNA, and water as a negative control. The thermal cycler program was as follows: 1 cycle of 96 °C for 3 min, then 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, followed by a cycle of 72 °C for 10 min and then held at 4 °C. The products were visualized on a 1% agarose gel, stained with ethidium bromide, and viewed under UV transillumination.

2.11.3 ITS region standard PCR

For the ITS region, 1055F forward primer designed from a conserved region of the 18S rRNA gene (Sogin, 1990) and ITSR reverse primer designed from a conserved region of the 28S rRNA gene (Aktas et al., 2007) (Table 4) were used to amplify the genomic region spanning the ITS1-5.8S gene-ITS2 region. Primary PCR reactions were prepared using 10 ng Hc-GRF gDNA, 30 ng Hc-H992 gDNA, and water as a negative control. The PCR was conducted in a thermal cycler programmed with the following conditions: 1 cycle of 96 °C for 3 min, then 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, followed by a cycle of 72 °C for 10 min, and then held at 4 °C.

Table 4
Primers for 18S rRNA gene and ITS genomic region amplification

Primer	Sequence (5´ to 3´)	T_A	Expected length
18S Primary PCR			
AN	GCTTGTCTTAAAGATTAAGCCATGC	60 °C	1727 bp
B	GATCCTTCTGCAGGTTCACCTAC		
ITS Primary PCR			
1055F	GGTGGTGCATGGCCG	55 °C	1981 bp
ITSR	GGTCCGTGTTTCAAGACGG		
ITS Nested PCR			
ITSF	GAGAAGTCGTAACAAGGTTTCCG	55 °C	872 bp
28SRN2	CGGGTAACCTCGCCTG		

The products were visualized on a 1% agarose gel and viewed under UV transillumination.

A nested PCR was performed for the ITS reactions using primers ITSF (located in the 18S rRNA gene) (Aktas et al., 2007) and 28SRN2 (designed from the 28S rRNA gene) and 1 µl of a 1:10 dilution of the primary PCR reactions as template (Table 4). The thermal cycler conditions were: 1 cycle of 96 °C for 3 min, then 30 cycles of 94 °C for 10 s, 55 °C for 10 s, 72 °C for 2 min, followed by a cycle of 72 °C for 10 min and then held at 4 °C. The products were visualized on a 1% agarose gel, stained with ethidium bromide, and viewed under UV transillumination.

2.11.4 Ligation and transformation

The Hc-GRF and Hc-H992 products from the 18S rRNA PCR and the nested ITS PCR were ligated using the TOPO® TA Cloning Kit and transformed into One Shot® competent *E. coli* cells (Invitrogen). Following the TOPO® protocol, 2-4 µl (approximately 600-800 ng) of each PCR product was ligated into the TOPO® vector, and then transformed into the competent cells. The samples were plated on LB agar with 50 µg/ml kanamycin and incubated in a 37 ºC incubator overnight.

2.11.5 Colony PCR

For each transformation, colonies were analyzed by PCR to determine if the gene fragment of interest was successfully incorporated into the vector. The reactions were performed using M13 forward and M13 reverse primers (located in the vector) and the following thermal cycler conditions: 1 cycle of 94 ºC for 10 min, then 30 cycles of 94 ºC for 30 s, 50 ºC for 30 s, and 72 ºC for 2 min, followed by a cycle of 72 ºC for 10 min, and then held at 4 ºC. The products were visualized on a 1% agarose gel and viewed under UV transillumination. Colonies that possessed the appropriate sized insert for each transformation were inoculated into 6 ml LB broth containing 6 µl ampicillin and incubated overnight in a 37 ºC shaker incubator at 200 rpm.

2.11.6 Plasmid DNA preparation

For select colonies, plasmid DNA (pDNA) for each of the samples was prepared using the QIAprep Spin Mini Kit (Qiagen Inc.,Valencia, California, USA). The bacteria

cells from the broth cultures were pelleted by centrifuging at 4 ℃ for 30 min at 720 X *g*. The pDNA was purified following the kit protocol and eluted in 50 µl of sterile water. The pDNA was visualized on a 1% agarose gel, stained with ethidium bromide, and viewed under UV transillumination to verify the presence of the insert. The concentration of the pDNA was determined using the NanoDrop Spectrophotometer.

2.11.7 Sequencing and analysis

The cloned Hc-H992 and Hc-GRF 18S and ITS genes were sequenced by Davis Sequencing (Davis, California, USA). The sequences were analyzed using Sequencher 3.11 software (Gene Codes Corporation, Inc., Ann Arbor, Michigan, USA). BLAST similarity searches (Altschul et al., 1990) were performed for all 18S rRNA gene and ITS region sequences obtained (GenBank database, National Center for Biotechnology Information, National Institutes of Health, Bethesda, Maryland, USA). The sequences were aligned and compared using the ClustalW 1.8 Program (EMBL-EBI, 2007).

3. RESULTS

3.1 Modified McMaster test and larval identification

For all of the *H. contortus* strains, the EPG determined that adequate quantities of eggs were present in the fecal sample for conducting the DR assay (Table 5). The larval identification determined that *H. contortus* was the only species of nematode present in all of the fecal samples.

Table 5
Results of the modified McMaster test and the larval identification for each strain of *H. contortus*

H. contortus Strain	EPG	Fecal Weight (grams)	Estimated egg count	Larval Identification
Hc-ES	4,266	26.80	114,329	100 % *H. contortus*
Hc-EG	300	5.02	1,506	100 % *H. contortus*
Hc-OS	900	13.40	12,060	100 % *H. contortus*
Hc-OG	4,200	8.90	37,380	100 % *H. contortus*
Hc-RFR	1,000	6.92	6,920	100 % *H. contortus*
Hc-H992	500	10.71	5,355	100 % *H. contortus*
Hc-GRF	16,700	224.70	3,752,490	100 % *H. contortus*

3.2 DrenchRite® assay

3.2.1 Hc-ES strain

The Hc-ES strain was susceptible to BZ/LEV combination and AVM with critical wells 4 and 2.5 respectively (Table 6; Fig. 2). The strain approached the weak resistance level to BZ with critical well 5. The critical well for LEV was well 7.5, which falls within the weakly resistant range. Due to the level of resistance to LEV, this strain was not included in the molecular analysis of the P-glycoprotein.

Table 6
The critical wells (Cw) and corresponding levels of resistance for the *H. contortus* strains as determined by the DrenchRite® assay. (S = susceptible, S / W = bordering on susceptible and weakly resistant, W = weakly resistant, W / R = bordering on weakly resistant and highly resistant, R = highly resistant)

	Suspected Susceptible			**Suspected Resistant**			
	Hc-ES	**Hc-EG**	**Hc-OS**	**Hc-OG**	**Hc-RFR**	**Hc-H992**	**Hc-GRF**
BZ	Cw = 5 S	Cw = 6 W	Cw = 4.5 S	Cw = 4.5 S	Cw = 7.5 W	Cw = 8.5 W / R	Cw = 11 R
LEV	Cw = 7.5 W	Cw = 8.5 W / R	Cw = 5.5 S / W	Cw = 5 S	Cw = 5.5 S / W	Cw = 5 S	Cw = 10.5 R
BZ / LEV	Cw = 4 S	Cw = 5 S	Cw = 4.5 S	Cw = 5 S	Cw = 6 W	Cw = 5.5 S / W	Cw = 8.5 W / R
AVM	Cw = 2.5 S	Cw = 5.5 S / W	Cw = 3 S	Cw = 3.5 S	Cw = 6.5 W	Cw = 6.5 W	Cw = 6.5 W

3.2.2 Hc-EG strain

The Hc-EG strain was susceptible to the BZ/LEV combination with a critical well of 5, which is nearing the weakly resistant range (Table 6; Fig. 2). The critical well for AVM was 5.5, which borders between the susceptible and weakly resistant ranges. For BZ, the critical well was determined to be well 6, which falls within the weakly resistant range and the LEV critical well was 8.5, which falls on the border between the weakly resistant and the highly resistant ranges. This strain was included in the Pgp molecular analysis because it demonstrated a higher level of resistance to LEV.

3.2.3 Hc-OS strain

The Hc-OS strain was susceptible to BZ, BZ/LEV combination and AVM, with critical wells 4.5, 4.5, and 3, respectively (Table 6; Fig. 2). The critical well for LEV was 5.5, which falls on the border between the susceptible and weakly resistant range. This strain was predominantly susceptible with the LEV bordering on weak resistance and was included in the molecular analysis.

3.2.4 Hc-OG strain

The Hc-OG strain was susceptible to all anthelmintics tested. The critical wells were as follows: well 4.5 for BZ, well 5 for LEV, well 5 for BZ/LEV combination, and well 3.5 for AVM (Table 6; Fig. 2). However, the levels of resistance to LEV, as well as the BZ/LEV combination, are approaching the weakly resistant level. This strain was

determined to be the most susceptible in this study and was included in the molecular analysis.

3.2.5 Hc-RFR strain

The Hc-RFR critical well for LEV was 5.5, which falls on the border between the susceptible and weakly resistant ranges (Table 6; Fig. 2). For the other anthelmintics tested, the critical wells were within the weakly resistant range and were as follows: well 7.5 for BZ, well 6 for BZ/LEV combination, and well 6.5 for AVM. This strain was determined to be predominantly weakly resistant, with LEV on the border between susceptible and weakly resistant and was included in the molecular analysis.

3.2.6 Hc-H992 strain

The Hc-H992 was susceptible for LEV with a critical well of 5, which is nearing the weakly resistant range (Table 6; Figure 2). The critical well was 5.5 for the BZ/LEV combination, which falls on the border between the susceptible and weakly resistant ranges. This strain was weakly resistant to AVM with a critical well of 6.5, but bordered on the weakly and highly resistant ranges for BZ with a critical well of 8.5. This strain was not as resistant as originally suspected, but still showed a level of resistance to BZ and AVM and was included in the molecular analysis.

3.2.7 Hc-GRF strain

The Hc-GRF strain was weakly resistant to AVM with a critical well of 6.5, but bordered on the weakly and highly resistant ranges for the BZ/LEV combination with a critical well of 8.5 (Table 6; Figure 2). This strain was highly resistant to BZ and LEV with critical wells of 11 and 10.5 respectively. This strain was determined to be the most resistant strain in the study, being either weakly or highly resistant to all of the anthelmintics tested and was included in the molecular analysis.

3.3 P-glycoprotein molecular analysis

None of the *H. contortus* strains showed transcription levels of Pgp at levels detectable in the PCR assay used in this study (represented by Fig. 4). Using the same amount of cDNA in the reactions, the housekeeping gene for each of the samples was amplified at detectable levels. The smaller amplicon of Pgp showed similar results.

3.4 Hc-GRF molecular characterization

3.4.1 18S rRNA

The 18S rRNA sequences obtained from isolates for Hc-H992 and Hc-GRF were compared to the sequences available in the GenBank database for *H. contortus* (accession no. L04153), *Haemonchus placei* (accession no. L04154) and *Haemonchus similis* (accession no. L04152) (Fig. 5). The three reference sequences were 100%

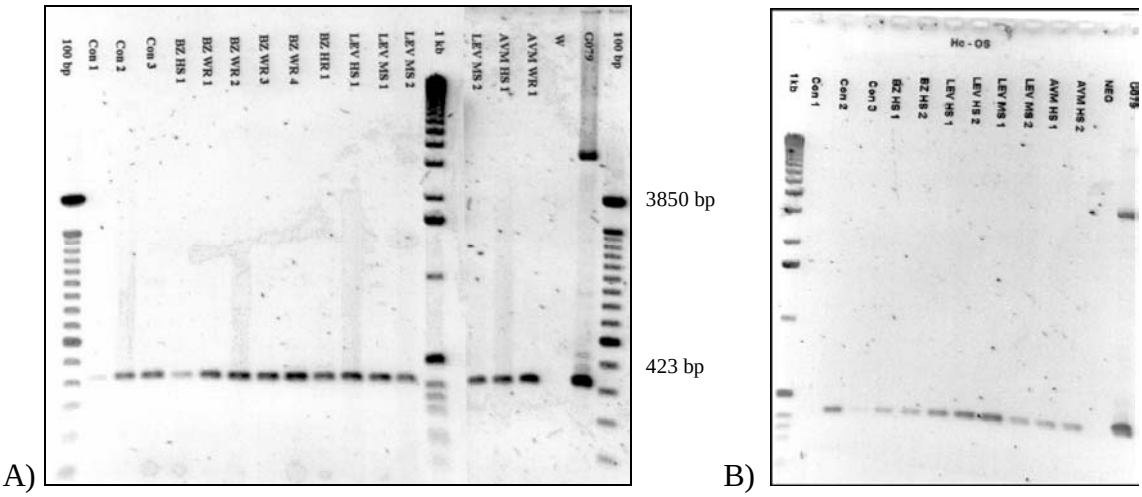

Fig. 4. A) Gel image of the P-glycoprotein analysis for the Hc-H992 strain. B) Gel image of the P-glycoprotein analysis for the Hc-OS strain. For both images: the Pgp positive (G079) and negative (water) are shown on the right of each gel at 3850bp and the housekeeping gene for each sample tested is shown at 450bp. There was not amplification of Pgp for the test samples. These gels are representative for all of the strains evaluated.

identical when aligned using a Lalign program (EMBNET.CH, 2007). The Hc-H992 consensus sequence showed an identity of 99.4% to *H. contortus, H. placei* and *H. similis* and 99.5% to the Hc-GRF. In comparison, the Hc-GRF showed a 99.9% identity to all three of the reference sequences. Several base differences (designated in bold below) occurred in the Hc-H992 isolates, however at position 1165 (↓), there was a nucleotide inserted for both Hc-H992 and Hc-GRF. Based on the similarity of the Hc-GRF sequences to the other known *Haemonchus* spp, it was confirmed to be a species of *Haemonchus*.

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                Primer AN →

L04153-Hc       GCTCAGTTTAAAGATTAAGCCATGCATGTCGAGTTCATCTTTGAAGAGAAACTGCGAACG 60
Hc-GRF-6        -----------AGATTAAGCCATGCATGTCGAGTTCATCTTTGAAGAGAAACTGCGAACG 49
Hc-GRF-8        GCTTGTCTTAAAGATTAAGCCATGCATGTCGAGTTCATCTTTGAAGAGAAACTGCGAACG 60
Hc-H992-2       GCTTGTCTTAAAGATTAAGCCATGCATGTCGAGTTCATCTTTGAAGAGAAACTGCGAACG 60
Hc-H992-3       GCTTGTCTTAAAGATTAAGCCATGCATGTCGAGTTCATCTTTGAAGAGAAACTGCGAACG 60
L04154-Hp       GCTCAGTTTAAAGATTAAGCCATGCATGTCGAGTTCATCTTTGAAGAGAAACTGCGAACG 60
L04152-Hs       GCTCAGTTTAAAGATTAAGCCATGCATGTCGAGTTCATCTTTGAAGAGAAACTGCGAACG 60
                ********************************************** 

L04153-Hc       GCTCATTAGAGCAGATGTCATTTATTCGGAACGTCCTTTTGGATAACTGCGGTAATTCTG 120
Hc-GRF-6        GCTCATTAGAGCAGATGTCATTTATTCGGAACGTCCTTTTGGATAACTGCGGTAATTCTG 109
Hc-GRF-8        GCTCATTAGAGCAGATGTCATTTATTCGGAACGTCCTTTTGGATAACTGCGGTAATTCTG 120
Hc-H992-2       GCTCATTAGAGCAGATGTCATTTATTCGGAACGTCCTTTTGGATAACTGCGGTAATTCTG 120
Hc-H992-3       GCTCATTAGAGCAGATGTCATTTATTCGGAACG**A**CCTTTTGGATAACTGCGGTAATTCTG 120
L04154-Hp       GCTCATTAGAGCAGATGTCATTTATTCGGAACGTCCTTTTGGATAACTGCGGTAATTCTG 120
L04152-Hs       GCTCATTAGAGCAGATGTCATTTATTCGGAACGTCCTTTTGGATAACTGCGGTAATTCTG 120
                ******************************** ************************ 

L04153-Hc       GAGCTAATACATGCAAATAAACCCTGACTTTTGAAAGGGTGCAATTATTAGAGCAAATCA 180
Hc-GRF-6        GAGCTAATACATGCAAATAAACCCTGACTTTTGAAAGGGTGCAATTATTAGAGCAAATCA 169
Hc-GRF-8        GAGCTAATACATGCAAATAAACCCTGACTTTTGAAAGGGTGCAATTATTAGAGCAAATCA 180
Hc-H992-2       GAGCTAATACATGCAAATAAACCCTGACTTTTGAAAGGGTGCAATTATTAGAGCAAATCA 180
Hc-H992-3       GAGCTAATACATGC**G**AATAAACCCTGACTTTTGAAAGGGTGCAATTATTAGAGCAAATCA 180
L04154-Hp       GAGCTAATACATGCAAATAAACCCTGACTTTTGAAAGGGTGCAATTATTAGAGCAAATCA 180
L04152-Hs       GAGCTAATACATGCAAATAAACCCTGACTTTTGAAAGGGTGCAATTATTAGAGCAAATCA 180
                ************** ****************************************** 

L04153-Hc       ATCACTTTCGGGTGCAGTTTGCTGACTCTGAATAACGCAGCATATCGGCGGCTTGTTCGC 240
Hc-GRF-6        ATCACTTTCGGGTGCAGTTTGCTGACTCTGAATAACGCAGCATATCGGCGGCTTGTTCGC 229
Hc-GRF-8        ATCACTTTCGGGTGCAGTTTGCTGACTCTGAATAACGCAGCATATCGGCGGCTTGTTCGC 240
Hc-H992-2       ATCACTTTCGGGTGCAGTTTGCTGACTCTGAATAACGCAGCATATCGGCGGCTTGTTCGC 240
Hc-H992-3       ATCACTTTCGGGTGCA**T**TTTGCTGACTCTGAATAACGCAGCATATCGGCGGCTTGTTCGC 240
L04154-Hp       ATCACTTTCGGGTGCAGTTTGCTGACTCTGAATAACGCAGCATATCGGCGGCTTGTTCGC 240
L04152-Hs       ATCACTTTCGGGTGCAGTTTGCTGACTCTGAATAACGCAGCATATCGGCGGCTTGTTCGC 240
                *************** ****************************************** 

L04153-Hc       CGATATTCCGAAAAAGTGTCTGCCCTATCAACCTGATGGTAGTCTATTAGTCTACCATGG 300
Hc-GRF-6        CGATATTCCGAAAAAGTGTCTGCCCTATCAACCTGATGGTAGTCTATTAGTCTACCATGG 289
Hc-GRF-8        CGATATTCCGAAAAAGTGTCTGCCCTATCAACCTGATGGTAGTCTATTAGTCTACCATGG 300
Hc-H992-2       CGATATTCCGAAAAAGTGTCTGCCCTATCAACCTGATGGTAGTCTATTAGTCTACCATGG 300
Hc-H992-3       CGATATTCCGAAAAAGTGTCTGCCCTATCAACCTGATGGTAGTCTATTAGTCTACCATGG 300
L04154-Hp       CGATATTCCGAAAAAGTGTCTGCCCTATCAACCTGATGGTAGTCTATTAGTCTACCATGG 300
L04152-Hs       CGATATTCCGAAAAAGTGTCTGCCCTATCAACCTGATGGTAGTCTATTAGTCTACCATGG 300
                ************************************************************ 

L04153-Hc       TTATTACGGGTAACGGAGAATAAGGGTTCGACTCCGGAGAGGGAGCCTTAGAAACGGCTA 360
Hc-GRF-6        TTATTACGGGTAACGGAGAATAAGGGTTCGACTCCGGAGAGGGAGCCTTAGAAACGGCTA 349
Hc-GRF-8        TTATTACGGGTAACGGAGAATAAGGGTTCGACTCCGGAGAGGGAGCCTTAGAAACGGCTA 360
Hc-H992-2       TTATTACGGGTAACGGAGAATAAGGGTTCGACTCCGGAGAGGGAGCCTTAGAAACGGCTA 360
Hc-H992-3       TTATTACGGGTAACGGAGAATAAGGGTTCGACTCCGGAGAGGGAGCCTTAGAAACGGCTA 360
L04154-Hp       TTATTACGGGTAACGGAGAATAAGGGTTCGACTCCGGAGAGGGAGCCTTAGAAACGGCTA 360
L04152-Hs       TTATTACGGGTAACGGAGAATAAGGGTTCGACTCCGGAGAGGGAGCCTTAGAAACGGCTA 360
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Fig. 5. The Hc-H992 and Hc-GRF 18S rRNA gene sequence alignment with denotation of primer location. The reference sequences are designated by the GenBank accession numbers as follows: L04153-Hc for *H. contortus*, L04154-Hp for *H. placei* and L04152-Hs for *H. similis*. Single base substitutions are designated in bold type. A single base insertion at position 1165 (↓) occurred in both the Hc-H992 and Hc-GRF sequences.

```
L04153-Hc       CCACATCCAAGGAAGGCAGCAGGCGCGAAACTTATCCAATCTTGAACAGATGAGATAGTG 420
Hc-GRF-6        CCACATCCAAGGAAGGCAGCAGGCGCGAAACTTATCCAATCTTGAACAGATGAGATAGTG 409
Hc-GRF-8        CCACATCCAAGGAAGGCAGCAGGCGCGAAACTTATCCAATCTTGAACAGATGAGATAGTG 420
Hc-H992-2       CCACATCCAAGGAAGGCAGCAGGCGCGAAACTTATCCAATCTTGAACAGATGAGATAGTG 420
Hc-H992-3       CCACATCCAAGGAAGGCAGCAGGCGCGAAACTTATCCAATCTTGAACAGATGAGATAGTG 420
L04154-Hp       CCACATCCAAGGAAGGCAGCAGGCGCGAAACTTATCCAATCTTGAACAGATGAGATAGTG 420
L04152-Hs       CCACATCCAAGGAAGGCAGCAGGCGCGAAACTTATCCAATCTTGAACAGATGAGATAGTG 420
                ************************************************************

L04153-Hc       ACTAAAAATAAAAAGACCATTCCTATGGAACGGTCATTTCAATGAGTTGATCATAAACCT 480
Hc-GRF-6        ACTAAAAATAAAAAGACCATTCCTATGGAACGGTCATTTCAATGAGTTGATCATAAACCT 469
Hc-GRF-8        ACTAAAAATAAAAAGACCATTCCTATGGAACGGTCATTTCAATGAGTTGATCATAAACCT 480
Hc-H992-2       ACTAAAAATAAAAAGACCATTCCTATGGAACGGTCATTTCAATGAGTTGATCATAAACCT 480
Hc-H992-3       ACTAAAAATAAAAAGACCATTCCTATGGAACGGTCATTTCAATGAGTTGATCATAAACCT 480
L04154-Hp       ACTAAAAATAAAAAGACCATTCCTATGGAACGGTCATTTCAATGAGTTGATCATAAACCT 480
L04152-Hs       ACTAAAAATAAAAAGACCATTCCTATGGAACGGTCATTTCAATGAGTTGATCATAAACCT 480
                ************************************************************

L04153-Hc       TTTTTCGAGGATCAAGTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCC 540
Hc-GRF-6        TTTTTCGAGGATCAAGTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCC 529
Hc-GRF-8        TTTTTCGAGGATCAAGTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCC 540
Hc-H992-2       TTTTTCGAGGATCAAGTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCC 540
Hc-H992-3       TTTTTCGAGGATCAAGTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCC 540
L04154-Hp       TTTTTCGAGGATCAAGTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCC 540
L04152-Hs       TTTTTCGAGGATCAAGTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCC 540
                ************************************************************

L04153-Hc       ACTAGTGTAAATCGTCATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGAGTTACATGC 600
Hc-GRF-6        ACTAGTGTAAATCGTCATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGAGTTACATGC 589
Hc-GRF-8        ACTAGTGTAAATCGTCATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGAGTTACATGC 600
Hc-H992-2       ACTAGTGTAAATCGTCATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGAGTTACATGC 600
Hc-H992-3       ACTAGTGTAAATCGTCATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGAGTTACATGC 600
L04154-Hp       ACTAGTGTAAATCGTCATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGAGTTACATGC 600
L04152-Hs       ACTAGTGTAAATCGTCATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGAGTTACATGC 600
                ************************************************************

L04153-Hc       AGTGATTCGCCTTTGGCGTTAATCGCTGTTGTAACTATTTGCTGGTTTTCTATTGAGGTT 660
Hc-GRF-6        AGTGATTCGCCTTTGGCGTTAATCGCTGTTGTAACTATTTGCTGGTTTTCTATTGAGGTT 649
Hc-GRF-8        AGTGATTCGCCTTTGGCGTTAATCGCTGTTGTAACTATTTGCTGGTTTTCTATTGAGGTT 660
Hc-H992-2       AGTGATTCGCCTTTGGCGTTAATCGCTGTTGTAACTATTTGCTGGTTTTCT**G**TTGAGGTT 660
Hc-H992-3       AGTGATTCGCCTTTGGCGTTAATCGC**A**GTTGTAACTATTTGCTGGTTTTCT**G**TTGAGGTT 660
L04154-Hp       AGTGATTCGCCTTTGGCGTTAATCGCTGTTGTAACTATTTGCTGGTTTTCTATTGAGGTT 660
L04152-Hs       AGTGATTCGCCTTTGGCGTTAATCGCTGTTGTAACTATTTGCTGGTTTTCTATTGAGGTT 660
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L04153-Hc       TCGGCTTCTTTAGTGGCTAGCGAGTTTACTTTGAATAAATTAGAGTGCTCAGAACAAGCG 720
Hc-GRF-6        TCGGCTTCTTTAGTGGCTAGCGAGTTTACTTTGAATAAATTAGAGTGCTCAGAACAAGCG 709
Hc-GRF-8        TCGGCTTCTTTAGTGGCTAGCGAGTTTACTTTGAATAAATTAGAGTGCTCAGAACAAGCG 720
Hc-H992-2       TCGGCTTCTTTAGTGGCTAGCGAGTTTACTTTGAATAAATTAGAGTGCTCAGAACAAGCG 720
Hc-H992-3       TCGGCTTCTTTAGTGGCTAGCGAGTTTACTTTGAATAAATTAGAGTGCTCAGAACAAGCG 720
L04154-Hp       TCGGCTTCTTTAGTGGCTAGCGAGTTTACTTTGAATAAATTAGAGTGCTCAGAACAAGCG 720
L04152-Hs       TCGGCTTCTTTAGTGGCTAGCGAGTTTACTTTGAATAAATTAGAGTGCTCAGAACAAGCG 720
                ************************************************************

L04153-Hc       TTTGCTTGAATGGTCGATCATGGAATAATAAAAGAGGACTTCGGTTCTATTTATTGGTTC 780
Hc-GRF-6        TTTGCTTGAATGGTCGATCATGGAATAATAAAAGAGGACTTCGGTTCTATTTATTGGTTC 769
Hc-GRF-8        TTTGCTTGAATGGTCGATCATGGAATAATAAAAGAGGACTTCGGTTCTATTTATTGGTTC 780
Hc-H992-2       TTTGCTTGAATGGTCGATCATGGAATAATAAAAGAGGACTTCGGTTCTATTTATTGGTTC 780
Hc-H992-3       TTTGCTTGAATGGTCGATCATGGAATAATAAAAGAGGACTTCGGTTCTATTTATTGGTTC 780
L04154-Hp       TTTGCTTGAATGGTCGATCATGGAATAATAAAAGAGGACTTCGGTTCTATTTATTGGTTC 780
L04152-Hs       TTTGCTTGAATGGTCGATCATGGAATAATAAAAGAGGACTTCGGTTCTATTTATTGGTTC 780
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Fig. 5. Continued.

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L04153-Hc       AGGAACTGAAATAATGGTTAAGAGGGACAATTCGGGGGCATTCGTATCCCTGCGCGAGAG 840
Hc-GRF-6        AGGAACTGAAATAATGGTTAAGAGGGACAATTCGGGGGCATTCGTATCCCTGCGCGAGAG 829
Hc-GRF-8        AGGAACTGAAATAATGGTTAAGAGGGACAATTCGGGGGCATTCGTATCCCTGCGCGAGAG 840
Hc-H992-2       AGGAACTGAAATAATGGTTAAGAGGGACAATTCGGGGGCATTCGTATCCCTGCGCGAGAG 840
Hc-H992-3       AGGAACTGAAATAATGGTTAAGAGGGACAATTCGGGGGCATTCGTATCCCTGCGCGAGAG 840
L04154-Hp       AGGAACTGAAATAATGGTTAAGAGGGACAATTCGGGGGCATTCGTATCCCTGCGCGAGAG 840
L04152-Hs       AGGAACTGAAATAATGGTTAAGAGGGACAATTCGGGGGCATTCGTATCCCTGCGCGAGAG 840
                ************************************************************

L04153-Hc       GTGAAATTCGTGGACCGCAGGGGGACGCCCTAAAGCGAAAGCATTTGCCAAGAATGTCTT 900
Hc-GRF-6        GTGAAATTCGTGGACCGCAGGGGGACGCCCTAAAGCGAAAGCATTTGCCAAGAATGTCTT 889
Hc-GRF-8        GTGAAATTCGTGGACCGCAGGGGGACGCCCTAAAGCGAAAGCATTTGCCAAGAATGTCTT 900
Hc-H992-2       GTGAAATTCGTGGACCGCAGGGGGACGCCCTAAAGCGAAAGCATTTGCCAAGAATGTCTT 900
Hc-H992-3       GTGAAATTCGTGGACCGCAGGGGGACGCCCTAAAGCGAAAGCATTTGCCAAGAATGTCTT 900
L04154-Hp       GTGAAATTCGTGGACCGCAGGGGGACGCCCTAAAGCGAAAGCATTTGCCAAGAATGTCTT 900
L04152-Hs       GTGAAATTCGTGGACCGCAGGGGGACGCCCTAAAGCGAAAGCATTTGCCAAGAATGTCTT 900
                ************************************************************

L04153-Hc       CATTAATCAAGAACGAAAGTCAGAGGTTCGAAGGCGATTAGATACCGCCCTAGTTCTGAC 960
Hc-GRF-6        CATTAATCAAGAACGAAAGTCAGAGGTTCGAAGGCGATTAGATACCGCCCTAGTTCTGAC 949
Hc-GRF-8        CATTAATCAAGAACGAAAGTCAGAGGTTCGAAGGCGATTAGATACCGCCCTAGTTCTGAC 960
Hc-H992-2       CATTAATCAAGAACGAAAGTCAGAGGTTCGAAGGCGATTAGATACCGCCCTAGTTCTGAC 960
Hc-H992-3       CATTAATCAAGAACGAAAGTCAGAGGTTCGAAGGCGATTAGATACCGCCCTAGTTCTGAC 960
L04154-Hp       CATTAATCAAGAACGAAAGTCAGAGGTTCGAAGGCGATTAGATACCGCCCTAGTTCTGAC 960
L04152-Hs       CATTAATCAAGAACGAAAGTCAGAGGTTCGAAGGCGATTAGATACCGCCCTAGTTCTGAC 960
                ************************************************************

L04153-Hc       CGTAAACTATGCCATCTAGCGATCCGATGGGGTATAGTTGCCTTGTCGAGGAGCTTCCCG 1020
Hc-GRF-6        CGTAAACTATGCCATCTAGCGATCCGATGGGGTATAGTTGCCTTGTCGAGGAGCTTCCCG 1009
Hc-GRF-8        CGTAAACTATGCCATCTAGCGATCCGATGGGGTATAGTTGCCTTGTCGAGGAGCTTCCCG 1020
Hc-H992-2       CGTAAACTATGCCATCTAGCGATCCGATGGGGTATAGTTGCCTTGTCGAGGAGCTTCCCG 1020
Hc-H992-3       CGTAAACTATGCCATCTAGCGATCCGATGGGGTATAGTTGCCTTGTCGAGGAGCTTCCCG 1020
L04154-Hp       CGTAAACTATGCCATCTAGCGATCCGATGGGGTATAGTTGCCTTGTCGAGGAGCTTCCCG 1020
L04152-Hs       CGTAAACTATGCCATCTAGCGATCCGATGGGGTATAGTTGCCTTGTCGAGGAGCTTCCCG 1020
                ************************************************************

L04153-Hc       GAAACGAAAGTCTTTCGGTTCCTGGGGTAGTATGGTTGCAAAGCTGAAACTTAAAGAAAT 1080
Hc-GRF-6        GAAACGAAAGTCTTTCCGTTCCTGGGGTAGTATGGTTGCAAAGCTGAAACTTAAAGAAAT 1069
Hc-GRF-8        GAAACGAAAGTCTTTCGGTTCCTGGGGTAGTATGGTTGCAAAGCTGAAACTTAAAGAAAT 1080
Hc-H992-2       GAAACGAAAGTCTTTCGGTTCCTGGGGTAGTATGGTTGCAAAGCTGAAACTTAAAGAAAT 1080
Hc-H992-3       GAAACGAAAGTCTTTCGGTTCCTGGGGTAGTATGGTTGCAAAGCTGAAACTTAAAGAAAT 1080
L04154-Hp       GAAACGAAAGTCTTTCGGTTCCTGGGGTAGTATGGTTGCAAAGCTGAAACTTAAAGAAAT 1080
L04152-Hs       GAAACGAAAGTCTTTCGGTTCCTGGGGTAGTATGGTTGCAAAGCTGAAACTTAAAGAAAT 1080
                *************** ****************************************

L04153-Hc       TGACGGAATGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAA 1140
Hc-GRF-6        TGACGGAATGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAA 1129
Hc-GRF-8        TGACGGAATGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAA 1140
Hc-H992-2       TGACGGAATGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAA 1140
Hc-H992-3       TGACGGAATGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAA 1140
L04154-Hp       TGACGGAATGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAA 1140
L04152-Hs       TGACGGAATGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAA 1140
                ************************************************************

                                        ↓
L04153-Hc       CTCACCCGGCCCGGACACCGTAAG-ATTGACAGATTGAAAGCTCTTTCTCGATTTGGTGG 1199
Hc-GRF-6        CTCACCCGGCCCGGACACCGTAAGGATTGACAGATTGAAAGCTCTTTCTCGATTTGGTGG 1189
Hc-GRF-8        CTCACCCGGCCCGGACACCGTAAGGATTGACAGATTGAAAGCTCTTTCTCGATTTGGTGG 1200
Hc-H992-2       CTCACCCGGCCCGGACACCGTAAGGATTGACAGATTGAAAGCTCTTTCTCGATTTGGTGG 1200
Hc-H992-3       CTCACCCGGCCCGGACACCGTAAGGATTGACAGATTGAAAGCTCTTTCTCGATTTGGTGG 1200
L04154-Hp       CTCACCCGGCCCGGACACCGTAAG-ATTGACAGATTGAAAGCTCTTTCTCGATTTGGTGG 1199
L04152-Hs       CTCACCCGGCCCGGACACCGTAAG-ATTGACAGATTGAAAGCTCTTTCTCGATTTGGTGG 1199
                ********************** ***********************************
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Fig. 5. Continued.

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L04153-Hc       TTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTTATTCCGATAACG 1259
Hc-GRF-6        TTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTTATTCCGATAACG 1249
Hc-GRF-8        TTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTTATTCCGATAACG 1260
Hc-H992-2       TTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTTATTCCGATAACG 1260
Hc-H992-3       TTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTTATTCCGATAACG 1260
L04154-Hp       TTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTTATTCCGATAACG 1259
L04152-Hs       TTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTTATTCCGATAACG 1259
                ************************************************************

L04153-Hc       AGCGAGACTCTAGCCTGCTAAATAGTGGCTGGATTTTTGAGTCCAGTCTACTTCTTAGAG 1319
Hc-GRF-6        AGCGAGACTCTAGCCTGCTAAATAGTGGCTGGATTTTTGAGTCCAGTCTACTTCTTAGAG 1309
Hc-GRF-8        AGCGAGACTCTAGCCTGCTAAATAGTGGCTGGATTTTTGAGTCCAGTCTACTTCTTAGAG 1320
Hc-H992-2       AGTGAGACTCTAGCCTGCTAAATAGTGGCTGGATTTTTGGGTCCAGTCTACTTCTTAGAG 1320
Hc-H992-3       AGCGAGACTCTAGCCTGCTAAATAGTGGCTGGATTTTTGAGTCCAGTCTACTTCTTAGAG 1320
L04154-Hp       AGCGAGACTCTAGCCTGCTAAATAGTGGCTGGATTTTTGAGTCCAGTCTACTTCTTAGAG 1319
L04152-Hs       AGCGAGACTCTAGCCTGCTAAATAGTGGCTGGATTTTTGAGTCCAGTCTACTTCTTAGAG 1319
                ** ********************************** ********************

L04153-Hc       GGATAAGCGGTGTTTAGCCGCACGAGATTGAGCGATAACAGGTCTGTGATGCCCTTAGAT 1379
Hc-GRF-6        GGATAAGCGGTGTTTAGCCGCACGAGATTGAGCGATAACAGGTCTGTGATGCCCTTAGAT 1369
Hc-GRF-8        GGATAAGCGGTGTTTAGCCGCACGAGATTGAGCGATAACAGGTCTGTGATGCCCTTAGAT 1380
Hc-H992-2       GGATAAGCGGTGTTTAGCCGCACGAGATTGAGCGATAACAGGTCTGTGATGCCCTTAGAT 1380
Hc-H992-3       GGATAAGCGGTGTTTAGCCGCACGAGATTGAGCGATAACAGGTCTGTGATGCCCTTAGAT 1380
L04154-Hp       GGATAAGCGGTGTTTAGCCGCACGAGATTGAGCGATAACAGGTCTGTGATGCCCTTAGAT 1379
L04152-Hs       GGATAAGCGGTGTTTAGCCGCACGAGATTGAGCGATAACAGGTCTGTGATGCCCTTAGAT 1379
                ************************************************************

L04153-Hc       GTCCGGGGCTGCACGCGCGCTACAATGGAAGAATCAGCTGGCCTATCCATTGCCGAAAGG 1439
Hc-GRF-6        GTCCGGGGCTGCACGCGCGCTACAATGGAAGAATCAGCTGGCCTATCCATTGCCGAAAGG 1429
Hc-GRF-8        GTCCGGGGCTGCACGCGCGCTACAATGGAAGAATCAGCTGGCCTATCCATTGCCGAAAGG 1440
Hc-H992-2       GTCCGGGGCTGCACGCGCGCTACAATGGAAGAATCAGCTGGCCTATCCATTGCCGAAAGG 1440
Hc-H992-3       GTCCGGGGCTGCACGCGCGCTACAATGGAAGAATCAGCTGGCCTATCCATTGCCGAAAGG 1440
L04154-Hp       GTCCGGGGCTGCACGCGCGCTACAATGGAAGAATCAGCTGGCCTATCCATTGCCGAAAGG 1439
L04152-Hs       GTCCGGGGCTGCACGCGCGCTACAATGGAAGAATCAGCTGGCCTATCCATTGCCGAAAGG 1439
                ************************************************************

L04153-Hc       CATTGGTAAACCGTTGAAACTCTTCCGTGACCGGGATAGGGAATTGTAATTATTTCCCTT 1499
Hc-GRF-6        CATTGGTAAACCGTTGAAACTCTTCCGTGACCGGGATAGGGAATTGTAATTATTTCCCTT 1489
Hc-GRF-8        CATTGGTAAACCGTTGAAACTCTTCCGTGACCGGGATAGGGAATTGTAATTATTTCCCTT 1500
Hc-H992-2       CATTGGTAAACCGTTGAAACTCTTCCGTGACCGGGATAGGGAATTGTAATTATTTCCCTT 1500
Hc-H992-3       CATTGGTAAACCGTTGAAACTCTTCCGTGACCGGGATAGGGAATTGTAATTATTTCCCTT 1500
L04154-Hp       CATTGGTAAACCGTTGAAACTCTTCCGTGACCGGGATAGGGAATTGTAATTATTTCCCTT 1499
L04152-Hs       CATTGGTAAACCGTTGAAACTCTTCCGTGACCGGGATAGGGAATTGTAATTATTTCCCTT 1499
                ************************************************************

L04153-Hc       GAACGAGGAATTCCTAGTAAGTGTGAGTCATCAGCTCACGCTGATTACGTCCCTGCCATT 1559
Hc-GRF-6        GAACGAGGAATTCCTAGTAAGTGTGAGTCATCAGCTCACGCTGATTACGTCCCTGCCATT 1549
Hc-GRF-8        GAACGAGGAATTCCTAGTAAGTGTGAGTCATCAGCTCACGCTGATTACGTCCCTGCCATT 1560
Hc-H992-2       GAACGAGGAATTCCTAGTAAGTGTGAGTCATCAGCTCACGCTGATTACGTCCCTGCCATT 1560
Hc-H992-3       GAACGAGGAATTCCTAGTAAGTGTGAGTCATCAGCTCACGCTGATTACGTCCCTGCCATT 1560
L04154-Hp       GAACGAGGAATTCCTAGTAAGTGTGAGTCATCAGCTCACGCTGATTACGTCCCTGCCATT 1559
L04152-Hs       GAACGAGGAATTCCTAGTAAGTGTGAGTCATCAGCTCACGCTGATTACGTCCCTGCCATT 1559
                ************************************************************

L04153-Hc       TGTACACACCGCCCGTCGCTGTCCGGGACTGAGCTGTCTCGAGAGGACTGCGGACTGCTG 1619
Hc-GRF-6        TGTACACACCGCCCGTCGCTGTCCGGGACTGAGCTGTCTCGAGAGGACTGCGGACTGCTG 1609
Hc-GRF-8        TGTACACACCGCCCGTCGCTGTCCGGGACTGAGCTGTCTCGAGAGGACTGCGGACTGCTG 1620
Hc-H992-2       TGTACACACCGCCCGTCGCTGTCCGGGACTGAGCTGTCTCGAGAGGACTGCGGACTGCTG 1620
Hc-H992-3       TGTACACACCGCCCGTCGCTGTCCGGGACTGAGCTGTCTCGAGAGGACTGCGGACTGCTG 1620
L04154-Hp       TGTACACACCGCCCGTCGCTGTCCGGGACTGAGCTGTCTCGAGAGGACTGCGGACTGCTG 1619
L04152-Hs       TGTACACACCGCCCGTCGCTGTCCGGGACTGAGCTGTCTCGAGAGGACTGCGGACTGCTG 1619
                ************************************************************
```

Fig. 5. Continued.

```
L04153-Hc       TATCGAGGCCTTCGGGTCGCGGTATGGCGGGAAACAGTTCAATCGCAATGGCTTGAACCG 1679
Hc-GRF-6        TATCGAGGCCTTCGGGTCGCGGTATGGCGGGAAACAGTTCAATCGCAATGGCTTGAACCG 1669
Hc-GRF-8        TATCGAGGCCTTCGGGTCGCGGTATGGCGGGAAACAGTTCAATCGCAATGGCTTGAACCG 1680
Hc-H992-2       TATCGAGGCCTTCGGGTCGCGGTATGGCGGGAAACAGTTCAATCGCAATGGCTTGAACCG 1680
Hc-H992-3       TATCGAGGCCTTCGGGTCGCGGTGTGGCGGGAAACAGTTCAATCGCAATGGCTTGAACCG 1680
L04154-Hp       TATCGAGGCCTTCGGGTCGCGGTATGGCGGGAAACAGTTCAATCGCAATGGCTTGAACCG 1679
L04152-Hs       TATCGAGGCCTTCGGGTCGCGGTATGGCGGGAAACAGTTCAATCGCAATGGCTTGAACCG 1679
                ********************** ************************************

                                                  ← Primer B

L04153-Hc       GGTAAAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGATGGATC 1726
Hc-GRF-6        GGTAAAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGAAGGATC 1716
Hc-GRF-8        GGTAAAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGAAGGATC 1727
Hc-H992-2       GGTAAAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGAAGGATC 1727
Hc-H992-3       GGTAAAAGTCGTAACAAGGTATCTGGAGGTGAACCTGCAGAAGGATC 1727
L04154-Hp       GGTAAAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGATGGATC 1726
L04152-Hs       GGTAAAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGATGGATC 1726
                ************************ ************** *****
```

Fig. 5. Continued.

3.4.2 ITS region

The ITS sequences obtained from isolates for Hc-H992 and Hc-GRF were compared to the sequences available in GenBank for *H. contortus* and *H. placei* ITS regions. There were no GenBank sequences available for *H. similis* to include in the comparisons.

The ITS 1 sequence in GenBank for *H. contortus* (accession no. AF044927) shows a 99.0% identity with *H. placei* (accession no. AF044929) with a total of 3 base substitution differences between the species. The Hc-H992 consensus sequence showed an identity of 97.5% to the Hc-GRF sequence, 100% to the *H. contortus* reference sequence and 99.0% to *H. placei.* In comparison, the Hc-GRF showed a 97.5% identity to *H. contortus* and a 97.0% identity to *H. placei.*

The sequences for the Hc-H992 and the Hc-GRF isolates contained a few minor differences (Fig. 6). Single base substitutions occurred in the Hc-H992 isolates as well

as in the Hc-GRF isolates (designated in bold type). There was a single base insertion at position 134 (↓) in only one of the Hc-H992 isolates and at position 270-273 (↓↓↓↓) there was a four base deletion in two of the Hc-GRF isolates and a single base deletion in one of the Hc-H992 isolates (position 270). All of the Hc-H992 and Hc-GRF sequences matched *H. contortus* in two out of the three nucleotide differences between *H. contortus* and *H. placei*. At the third position, two of the Hc-H992 isolates and one of the Hc-GRF isolates matched with *H. contortus* while the other three isolates matched with *H. placei*. The Hc-H992 and the Hc-GRF are most likely *H. contortus* based on these results.

```
AF044927-Hc       TCGAAACCTAAACACAAGGTTCCTTTGATCACGAGAAACCAACAGCTATGTTTTACGACT 60
6-GRF-ITS1        TCGAAACCTAAACACAAGGTTCCTTTGATCACGAGAAACCAACAGCTATGTTTTACGACT 60
7-GRF-ITS1        TCGAAACCTAAACACAAGGTTCCTTTGATCACGAGAAACCAACAGCTATGTTTTACGACT 60
10-GRF-ITS1       TCGAAACCTAAACACAAGGTTCCTTTGATCACGAGAAACCAACAGCTATGTTTTACGACT 60
9-H992-ITS1       TCGAAACCTAAACACAAGGTTCCTTTGATCACGAGAAACCAACAGCTATGTTTTACGACT 60
12-H992-ITS1      TCGAAACCTAAACACAAGGTTCCATTGATCACGAGAAACCAACAACTATGTTTTACGACT 60
14-H992-ITS1      TCGAAACCTGAACACAAGGTTCCTTTGATCACGAGAAACCAACAGCTATGTTTTACGACT 60
AF044929-Hp       TCGAAACCTAAACACAAGGTTCCTTTGATCACGAGAAACCAACAGCTATGTTTTACGACT 60
                  ********* ************* ******************** ***************

AF044927-Hc       TTGTCGTAAAAGTTGGGAGTATCACCCCCGTTAAAGCTCTATTACATGAGGTGTCTATGT 120
6-GRF-ITS1        TTGTCGTACAAGTTGGGAGTATCACCCCCGTTAAAGCTCTATTACATGAGGTGTCTATGT 120
7-GRF-ITS1        TTGTCGTAAAAGTTGGGAGTATCACCCCCGTTAAAGCTCTATTACATGAGGTGTCTATGT 120
10-GRF-ITS1       TTGTCGTACAAGTTGGGAGTATCACCCCCGTTAAAGCTCTATTACATGAGGTGTCTATGT 120
9-H992-ITS1       TTGTCGTAAAAGTTGGGAGTATCACCCCCGTTAAAGCTCTATTACATGAGGTGTCTATGT 120
12-H992-ITS1      TTGTCGTAAAAGTTGGGAGTCTCACCCCCGTTAAAGCTCTATTACATGAGGTGTCTATGT 120
14-H992-ITS1      TTGTCGTAAAAGTTGGGAGTATCACCCCCGTTAAAGCTCTATTACATGAGGTGTCTATGT 120
AF044929-Hp       TTGTCGTAAAAGTTGGGAGTATCACCCCCGTTAAAGCTCTATTACMTGAGGTGTCTATGT 120
                  ******** *********** ************************ **************
```

Fig. 6. The Hc-H992 and Hc-GRF ITS 1 alignment with single base substitutions designated in bold type. The reference sequences are designated by the GenBank accession numbers as follows: AF044927-Hc for *H. contortus* and AF044929-Hp for *H. placei*. A single base insertion at position 134 (↓) occurred in both the Hc-H992 and Hc-GRF isolates. In two of the Hc-GRF isolates, a four base deletion occurred at position 270-273 and a single base deletion occurred in one of the Hc-H992 isolates at position 270 (↓↓↓↓). The Hc-H992 and Hc-GRF isolates matched two out of three substitutions between *H. contortus* and *H. placei* (▼).

```
                          ↓                                                  ▼
AF044927-Hc       ATGACATGAGCCG-TTCGAGAGTGGCGGCTGTGATTGTTCATGCGAAGTTCCTATCATTG 179
6-GRF-ITS1        ATGACATGAGCCG-TTCAAGAGTGGTGGCTGTGATTGTTCATGCGAAGTTCCTATCATTG 179
7-GRF-ITS1        ATGACATGAGCCG-TTCGAGAGTGGCGGCTGTGATTGTTCATGCGAAGTTCCTATCATTG 179
10-GRF-ITS1       ATGACATGAGCCG-TTCAAGAGTGGTGGCTGTGATTGTTCATGCGAAGTTCCTATCATTG 179
9-H992-ITS1       ATGACATGAGCCG-TTCGAGAGTGGCGGCTGTGATTGTTCATGCGAAGTTCCTATCATTG 179
12-H992-ITS1      ATGACACGAGCCGCTTCAAGAGTGGCGGCTGTGATTGTTCATGCGAAGTTCCTATCATTG 180
14-H992-ITS1      ATGACATGAGCCG-TTCGAGAGTGGCGGCTGTGATTGTTCATGCGAAGTTCCTATCATTG 179
AF044929-Hp       ATGACATGAGCCG-TTCGAGAGTGGCGGCTGTGATTGTTCATGCGAAGTTCCTATCAGTG 179
                  ****** ****** *** ******* ****************************** **

                     ▼                                    ▼
AF044927-Hc       ATGGTTGAGCTTGAGACTTAATAAGTATTGCTATAATACTGCCTCACCGTTTATTAATGG 239
6-GRF-ITS1        ATGGTTGAGCTTGAGACTTAATAAGTATTGCTATAATACTGCCTCGCCGTTTATTAATGG 239
7-GRF-ITS1        ATGGTTGAGCTTGAGACTTAATAAGTATTGCTATAATACTGCCTCACCGTTTATTAATGG 239
10-GRF-ITS1       ATGGTTGAGCTTGAGACTTAATAAGTATTGCTATAATACTGCCTCGCCGTTTATTAATGG 239
9-H992-ITS1       ATGGTTGAGCTTGAGACTTAATAAGTATTGCTATAATACTGCCTCACCGTTTATTAATGG 239
12-H992-ITS1      ATGGTTGAGCTTGAGACTTAATAAGTATTGCTATAATACTGCCTCGCCGTTTATTGATGG 240
14-H992-ITS1      ATGGTTGAGCTTGAGACTTAATAAGTATTGCTATAATACTGCCTCACCGTTTATTAATGG 239
AF044929-Hp       ATAGTTGAGCTTGAGACTTAATAAGTATTGCTATAATACTGCCTCGCCGTTTATTAATGG 239
                  ** ************************************** ********* ****

                                          ↓↓↓↓
AF044927-Hc       TGGTTAAGTACGAACCAAATTACTTCTTGAAGTATGTGGTGTACTGTACCCGATTATATC 299
6-GRF-ITS1        TGGTTAAGTACGAACCAAATTACTTCTTGA----TGTGGTGTACTGTACCCGATTATATC 295
7-GRF-ITS1        TGGTTAAGTACGAACCAAATTACTTCTTGAAGTATGTGGTGTACTGTACCCGATTATATC 299
10-GRF-ITS1       TGGTTAAGTACGAACCAAATTACTTCTTGA----TGTGGTGTACTGTACCCGATTATATC 295
9-H992-ITS1       TGGTTAAGTACGAACCAAATTACTTCTTGAAGTATGTGGTGTACTGTACCCGATTATATC 299
12-H992-ITS1      TGGTTAAGTACGAACCAAATTACTTCTTGA-GTATGTGGTGTACTGTACCCGATTATATC 299
14-H992-ITS1      TGGTTAAGTACGAACCAAATTACTTCTTGAAGTATGTGGTGTACTGTACCCGATTATATC 299
AF044929-Hp       TGGTTAAGTACGAACCAAATTACTTCTTGAAGTATGTGGTGTACTGTACCCGATTATATC 299
                  ****************************    ************************

AF044927-Hc       GGGGAACCTTAATGATCACGCGTAGACGCCATTATAAAACACAAACATTCATTTTTACAG 359
6-GRF-ITS1        GGGGAACCTTAATGATCATGCGTAGACGCCATTGTAAAACACAAACATTCATTTTTACAG 355
7-GRF-ITS1        GGGGAACCTTAATGATCACGCGTAGACGCCATTATAAAACACAAACATTCATTTTTACAG 359
10-GRF-ITS1       GGGGAACCTTAATGATCATGCGTAGACGCCATTGTAAAACACAAACATTCATTTTTACAG 355
9-H992-ITS1       GGGGAACCTTAATGATCACGCGTAGACGCCATTATAAAACACAAACATTCATTTTTACAG 359
12-H992-ITS1      GGGGAACCTTAATGATCATGCGTAGACGCCATTATAAAACACAAACATTCATTTTTACAG 359
14-H992-ITS1      GGGGAACCTTAATGATCACGCGTAGACGCCATTATAAAACACAAACATTCATTTTTACAG 359
AF044929-Hp       GGGGAACCTTAATGATCACGCGTAGACGCCATTATAAAACACAAACATTCATTTTTACAG 359
                  ***************** ************** ************************

AF044927-Hc       TTTGCAGAACTTAGTGTTCACATTCATTTGTGTCACAAATATCGA 404
6-GRF-ITS1        TTTGCAGAACTTAGTGTTCACATTCATTTGTGTCACAAATATCGA 400
7-GRF-ITS1        TTTGCAGAACTTAGTGTTCACATTCATTTGTGCCACAAATATCGA 404
10-GRF-ITS1       TTTGCAGAACTTAGTGTTCACATTCATTTGTGTCACAAATATCGA 400
9-H992-ITS1       TTTGCAGAACTTAGTGTTCACATTCATTTGTGTCACAAATATCGA 404
12-H992-ITS1      TTTGCAGAACTTAGTGTTCACATTCATTTGTGTCACAAATATCGA 404
14-H992-ITS1      TTTGCAGAACTTAGTGTTCACATTCATTTGTGTCACAAATATCGA 404
AF044929-Hp       TTTGCAGAACTTAGTGTTCACATTCATTTGTGTCACAAATATCGA 404
                  ******************************* ************
```

Fig. 6. Continued.

The ITS 2 sequence in GenBank for *H. contortus* (accession no. AY647245) showed a 94.9% and 94.5% identity with *H. placei* (accession nos. X78812 and AJ577466 (99.6% identity)). The consensus sequence for Hc-H992 showed an identity of 96.5% to the Hc-GRF sequence, 97.0% to *H. contortus,* 97.8% to *H. placei* (X78812) and 97.4% to *H. placei* (AJ577466). The consensus sequence for the Hc-GRF showed an identity of 93.6% to *H. contortus,* 96.1% to *H. placei* (X78812) and 95.7% to *H. placei* (AJ577466).

Differences occurred in both the Hc-H992 isolates and the Hc-GRF isolates (Fig. 7). At position 32 (↓), there is a single base insertion in two of the Hc-GRF isolates and one of the Hc-H992 isolates. There was a six base deletion in the Hc-H992 and Hc-GRF isolates at position 95-100 (↓↓↓↓↓↓) compared to the *H. contortus* reference sequence, which is similar to *H. placei*. At positions 129 and 202 (†), some of the Hc-H992 and Hc-GRF isolates matched with the *H. placei* sequence. These positions were variable within *H. placei* isolates with two bases, T/C and T/A substituted at these positions; therefore the differences were not significant (Stevenson et al., 1995). Stevenson et al. (1995) demonstrated that the ITS 2 sequence for *H. contortus* differed by only three bases (▼) from *H. placei*. The sequences for all of the Hc-H992 and Hc-GRF isolates matched the sequence for *H. contortus* at these positions. Therefore, both isolates are most likely *H. contortus*.

```
                                                  ↓   ▼
AY647245-Hc       ----------AACCATATACTACAATGTGG-CTAATTTCAACATTGTTTGTCAAATGGC 48
6-GRF-ITS2        ----------AACCATATACTACAATGAGGGC-AATTTCAACATTGTTTGTCAAATGGC 48
7-GRF-ITS2        ----------AACCATATACTACAATGTGG-CTAATTTCAACATTGTTTGTCAAATGGC 48
10-GRF-ITS2       ----------AACCATATACTACAATGAGGGC-AATTTCAACATTGTTTGTCAAATGGC 48
9-H992-ITS2       ----------AACCATATACTACAATGTGG-CTAATTTCAACATTGTTTGTCAAATGGC 48
12-H992-ITS2      ----------AACCATATACTACAATGAGGGCTAATTTCAACATTGTTTGTCAAATGGC 49
14-H992-ITS2      ----------AACCATATACTACAATGTGG-CTAATTTCAACATTGTTTGTCAAATGGC 48
X78812-Hp         ----------AACCATATACTACAATGTGG-CTAGTTTCAACATTGTTTGTCAAATGGC 48
AJ577466-Hp       TCAGGGTTGTTAACCATATACTACAATGTGG-CTAGTTTCAACATTGTTTGTCAAATGGC 59
                            ***************** ** * * ************************

                                                                ↓↓↓↓↓↓
AY647245-Hc       ATTTGTCTTTTAGACAATTCCCATTTCAGTTCAAGAACATATACATATACATGCAACGTG 108
6-GRF-ITS2        ATTTGTCTTTGAGATAATTCCCATTTCAGCTCAAGAACATATACAT------GCAACGTG 102
7-GRF-ITS2        ATTTGTCTTTTAGACAATTCCCATTTCAGTTCAAGAACATATACAT------GCAACGTG 102
10-GRF-ITS2       ATTTGTCTTTGAGATAATTCCCATTTCAGCTCAAGAACATATACAT------GCAACGTG 102
9-H992-ITS2       ATTTGTCTTTTAGACAATTCCCATTTCAGTTCAAGAACATATACAT------GCAACGTG 102
12-H992-ITS2      ATTTGTCTTTAAGACAATTCCCATTTCAGTTCAAGAACATATACAT------GCAACGTG 103
14-H992-ITS2      ATTTGTCTTTTAGACAATTCCCATTTCAGTTCAAGAACATATACAT------GCAACGCG 102
X78812-Hp         ATTTGTCTTTTAGACAATTCCCATTTCAGTTCAAGAACATATACAT------GCAACGTG 102
AJ577466-Hp       ATTTGTCTTTTAGACATTTCCCATTTCAGTTCAAGAACATATACAT------GCAACGTG 113
                  ********** *** * ************ ****************      ****** *

                                  †
AY647245-Hc       ATGTTATGAAATTGTAACATTCCTGAATGATNTGAACATGTTGCCACTATTTGAGTGTAC 168
6-GRF-ITS2        ATGTTATGAAATTGTAACATCCCTGAATGATATGAACATGTTGCCACTATTTGAGTGTAC 162
7-GRF-ITS2        ATGTTATGAAATTGTAACATTCCTGAATGATATGAACATGTTGCCACTATTTGAGTGTAC 162
10-GRF-ITS2       ATGTTATGAAATTGTAACATCCCTGAATGATATGAACATGTTGCCACTATTTGAGTGTAC 162
9-H992-ITS2       ATGTTATGAAATTGTAACATTCCTGAATGATATGAACATGTTGCCACTATTTGAGTGTAC 162
12-H992-ITS2      ATGTTATGAAATTGTAACATCCCTGAATGATATGAACATGTTGCCACTATTTGAGTGTAC 163
14-H992-ITS2      ATGTTATGAAATTGTAACATTCCTGAATGATATGAACATGTTGCCACTATTTGAGTGTAC 162
X78812-Hp         ATGTTATGAAATTGTAACATCCCTGAATGATATGAACATGTTGCCACTATTTGAGTGTAC 162
AJ577466-Hp       ATGTTATGAAATTGTAACATCCCTGAATGATATGAACATGTTGCCACTATTTGAGTGTAC 173
                  ******************** ********** ****************************

                                                  †         ▼           ▼
AY647245-Hc       TCAGCGAATATTGAGATTGACTTAGATAGTGACTTGTATGGCGACGATGTTCTTTTATCA 228
6-GRF-ITS2        TCAGCGAATATTGAGATTGACTTAGATAGTGACATGTATGGCGACGATGTTCTTTTATCA 222
7-GRF-ITS2        TCAGCGAATATTGAGATTGACTTAGATAGTGACTTGTATGGCGACGATGTTCTTTTATCA 222
10-GRF-ITS2       TCAGCGAATATTGAGATTGACTTAGATAGTGACATGTATGGCGACGATGTTCTTTTATCA 222
9-H992-ITS2       TCAGCGAATATTGAGATTGACTTAGATAGTGACTTGTATGGCGACGATGCTCTTTTATCA 222
12-H992-ITS2      TCAGCGAATATAGAGAT-GACCTAGATAGTGACATGTATAGCGACGATGTTCTTTTATCA 222
14-H992-ITS2      TCAGCGAATATTGAGATTGACTTAGATAGTGACTTGTATGGCGACGATGTTCTCTTATCA 222
X78812-Hp         TCAGCGAATATTGAGATTGACTTAGATAGTGACATGTATGGCAACGATGTTCTTTTGTCA 222
AJ577466-Hp       TCAGCGAATATTGAGATTGACTTAGATAGTGACATGTATGGCAACGATGTTCTTTTGTCA 233
                  *********** ***** *** *********** ***** ** ****** *** ** ***
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Fig. 7. The Hc-H992 and Hc-GRF ITS 2 alignment with single base substitutions designated in bold type. The reference sequences are designated by the GenBank accession numbers as follows: AY647245-Hc for *H. contortus* and X78812-Hp and AJ577466-Hp for *H. placei*. A single base insertion at position 32 (↓) occurred in one of the Hc-H992 isolates and two of the Hc-GRF isolates. In all of the Hc-H992 and Hc-GRF isolates, a six base deletion occurred at position 95-100 (↓↓↓↓↓↓) which matches with *H. placei*. At positions 129 and 202 (†), the Hc-H992 and Hc-GRF isolates match with *H. placei,* however these are areas of variable bases. The Hc-H992 and Hc-GRF isolates matched *H. contortus* at the three bases where *H. contortus* differs from *H. placei* (▼) as determined be Stevenson et al., 1995.

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AY647245-Hc       TTTGTATAA--------------------------- 237
6-GRF-ITS2        TTTGTATAATGCAACCTGAGCTCAGGCGAGGTTACCC 259
7-GRF-ITS2        TTTGTATAATGCAACCTGAGCTCAGGCGAGGTTACCC 259
10-GRF-ITS2       TTTGTATAATGTAACCTGAGCTCAGGCGAGGTTACCC 259
9-H992-ITS2       TTTGTATAATGCAACCTGAGCTCAGGCGAGGTTACCC 259
12-H992-ITS2      TTTGTATAATGCAACCTGAGCTCAGGCGAGGTTACCC 259
14-H992-ITS2      TTTGTATAATGCAACCTGAGCTCAGGCGAGGTTACCC 259
X78812-Hp         TTTGTATAA--------------------------- 231
AJ577466-Hp       TTTGTATAATGCAACCTGAGCTCAGGCGTGATTACCC 270
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Fig. 7. Continued.

4. DISCUSSION

Seven isolates of *H. contortus* were evaluated for anthelmintic resistance at the outset of this study. The isolates were passaged through experimental sheep and/or goats, the eggs recovered and allowed to develop to larvae for worm identification. The results of the larval identification confirmed the recovery of the Texas strains of *H. contortus.* These were the expected results since the parasites had been administered to known helminth-free sheep/goats housed in a controlled environment and the feces had been collected directly from the rectum. Only *H. contortus* was identified in the giraffe sample, although it was anticipated that the giraffe would have a multiple infection based upon the condition of the feces and since the host was not housed in a controlled environment. It was also expected that free-living nematodes would be found in the giraffe fecal sample since the feces were collected from the ground, but none were present in the sample evaluated.

At the onset of this study, it was anticipated that resistant and susceptible strains of *H. contortus* would be acquired for molecular comparison. However, acquiring a completely susceptible strain of *H. contortus* proved to be a challenge in this study due to the extensive use of anthelmintics and the ensuing development of resistance. Of the suspected susceptible strains evaluated, only one, Hc-OG, was determined to be susceptible to all of the anthelmintics. The other strains were susceptible to at least one of the anthelmintics, but were bordering on or were weakly resistant to the others.

As for the resistant strains, the Hc-RFR was believed to be highly resistant to all of the anthelmintics and the Hc-H992 was believed to be highly resistant to

benzimidazoles. However, the DrenchRite® assay determined both of these strains to be only moderately resistant to the anthelmintics evaluated. Fortunately, the submission of a giraffe fecal sample to the Texas A&M University Diagnostic Parasitology Laboratory for evaluation provided us with a highly resistant *Haemonchus* strain for inclusion in this study.

This sample was from a young, male giraffe that had recently been acquired by the Florida zoo from a zoo in New Jersey. Upon arriving in Florida, an initial fecal sample was submitted to the Texas A&M University diagnostic laboratory. The EPG was 850 and the larvae were identified as *H. contortus* and several free-living nematode species. A DrenchRite® assay was performed and the results were inconclusive, possibly due to residual anthelmintics given prior to sample collection.

The giraffe was then successfully treated with ivermectin in conjunction with fenbendazole by the zoo and eventually introduced into the resident giraffe population. Initially, the giraffe was placed in a pasture with a larger population, but was quickly relocated to a smaller pasture with a feeder group consisting of four giraffes (three castrated males and one intact female). Approximately 2 months later, the giraffe presented with diarrhea and a fecal sample was submitted to Texas A&M University for evaluation. The EPG at this time was 16,700; the larvae were identified as *H. contortus*, and the DrenchRite® assay demonstrated resistance to all of the anthelmintics.

The young giraffe most likely acquired the highly resistant *H. contortus* infection while in the feeder group. Other giraffes that were previously placed in this group have also shown signs of severe infection (two of which died due to a heavy parasitemia).

Unfortunately, due to the resistance to all of the anthelmintics, the options available to treat *H. contortus* infections are limited. However, the zoo was successful in treating the young giraffe with a topical dose of moxidectin in conjunction with fenbendazole, followed 15 d later with a dose of ivermectin and ending with a second dose of topical moxidectin 27 d later. The use of these drugs in combination most likely enabled the clearing of the infection.

Upon reviewing the deworming schedule implemented by the zoo, it is clear how the *H. contortus* developed resistance to all three classes of anthelmintics. For more than 5 years, pyrantel tartrate (levamisole-like in activity) was administered daily in the feed, while ivermectin, fenbendazole and albendazole were rotated on a monthly basis. This allowed for the elimination of the highly susceptible parasites but did not allow enough time for the removal of the more resistant parasites. Therefore, only highly resistant parasites were present to reproduce, which compounded the problem and contributed to even stronger resistance development.

Transcription of Pgp was not detected in any larval worms in this study, whether exposed or not to anthelmintics, and even the smaller Pgp amplicon, which was located at the 3´ end of the gene sequence, was not detected. Unfortunately, the quality of the total RNA was not determined due to small sample volume and may have been a factor in the outcome of this study.

Our results would suggest that Pgp efflux is not involved in anthelmintic resistance in this stage of *H. contortus*. However, its importance in anthelmintic resistance should not be ruled out. P-glycoprotein is encoded for by at least seven

different genes which allows for a considerable amount of variability between the different isoforms. In this study, primers for the amplification of Pgp were designed from a sequence derived from adult *H. contortus* worms, which at the start of the study was the only complete sequence available in GenBank. However, this may not be the sequence of the Pgp isoform transcribed in the larval stage and not every Pgp isoform confers resistance (Sangster et al., 1999).

The mechanism of resistance to all three classes of anthelmintics may be explained by the overexpression of Pgp due to its ability to bind a wide range of substrates. The binding affinity of Pgp may be important in eliminating toxins from the parasites. However, the primary target of anthelmintics is the adult parasite and there may be stage-related differences in the expression of Pgp (Geary et al., 1999). There are also a number of other possible resistance mechanisms that may be employed by the larvae to combat the effects of anthelmintics.

Currently, there are three species of *Haemonchus* (*H. contortus*, *H. placei* and *H. similis*) that have been identified in North American ruminants (Lichtenfels et al., 1994). Both *H. placei* and *H. similis* are predominately found in cattle, while *H. contortus* is found in sheep, goats and many other domestic and wild ruminants. There is some controversy over whether these are indeed separate species. Morphologically, these species are slightly different, so molecular analysis has been conducted to determine species specific differences. Stevenson et al., (1995) identified only three single base differences between the ITS 2 region of *H. contortus* and *H. placei*.

The 18S rRNA gene sequence from the nematode acquired from the giraffe shared 99.9% identity with each of three *Haemonchus* species (GenBank accession nos. L04153, L04154 and L04152). This genus identity was supported by the 5.8S rRNA gene sequence analysis which matched the sequence for *H. contortus* (GenBank accession no. AY190133-5).

The analysis of the ITS 1 sequence showed that the parasite had a slightly higher identity (97.5%) to *H. contortus* than to *H. placei* (97.0%). There were three base differences identified in ITS 1 sequences previously reported from *H. contortus* (GenBank accession no. AF044927) and *H. placei* (GenBank accession no. AF044929) in this study. However, all three do not appear to be defining differences. Although all of the sequences for the Hc-H992 and Hc-GRF isolates matched two of these positions with *H. contortus,* the third position was variable with three of the isolates (one Hc-H992 and 2 Hc-GRF) matching *H. contortus* while the other three matched *H. placei.*

The ITS 2 sequence was more similar to *H. placei* (96.1% and 95.7%) than to *H. contortus* (93.6%). However, based on the 3 base differences between *H. contortus* and *H. placei* as described by Stevenson et al. (1995), the species from the giraffe is most likely *H. contortus* due to a 100% identity at these positions. Additional isolates should be evaluated to determine if there is truly a lack of variability in bases at these positions.

5. CONCLUSION

Haemonchus contortus continues to be a problematic parasite in the sheep and goat industry due to its increased resistance to anthelmintics. As is evidenced by the case of the giraffe, *H. contortus* is also becoming a problem in zoo settings where a number of ruminant species are at risk of acquiring this devastating parasite. Although Pgp transcription was not evident in the larvae in this study, the possible role of Pgp isoforms should not be ruled out in anthelmintic resistance in *H. contortus.*

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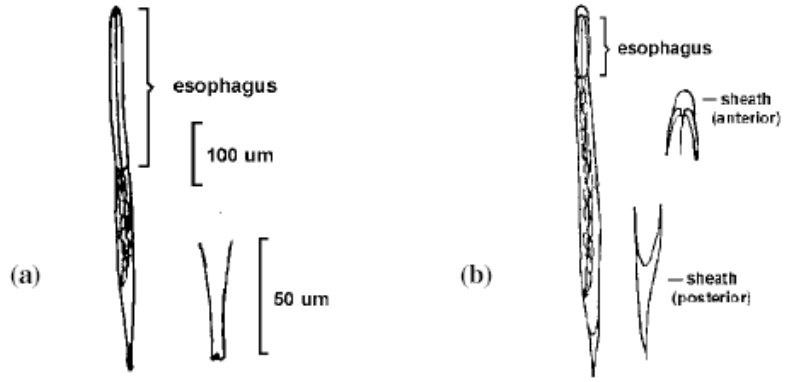
APPENDIX A

cgw updated 5/02

KEY FOR THE IDENTIFICATION OF THE 3RD STAGE LARVAE OF SOME COMMON GASTRO-INTESTINAL NEMATODES OF SHEEP

1. a. Esophagus rhabditiform ..... **free living nematode**
   b. Esophagus not rhabditiform **2**

2. a. Without sheath, esophagus nearly ½ the length of the body, L3 with notched tail ..... ***Strongyloides***
   b. With sheath, esophagus less than 1/4 the length of the body, tapered posterior **3**



3 a. Tail of sheath short (less than 50 um) or medium length (more than 50 um & less than 100 um) **4**
   b. Tail of sheath very long (greater than 100 um) **7**

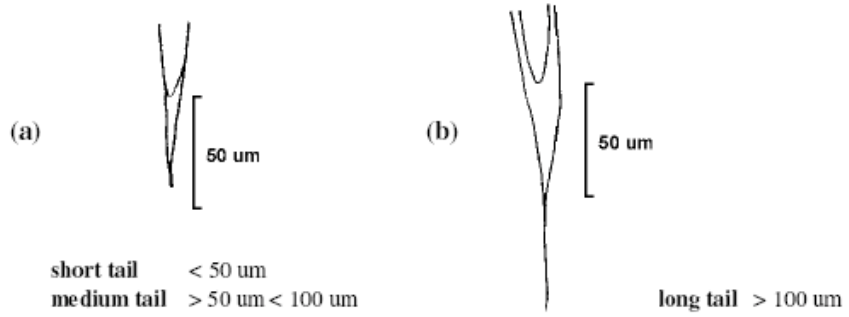
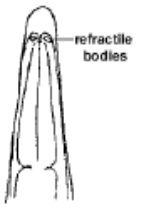


Fig. A.1 The L_3 larval identification key modified from The Manual of Veterinary Parasitological Laboratory Techniques, 1977.

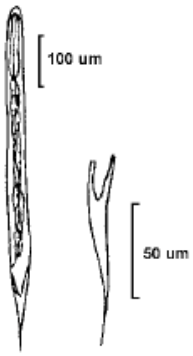
4. a. Two refractive bodies or a bright transverse band visible between buccal cavity and esophagus ..... ***Cooperia***
   b. Refractive bodies or band absent **5**



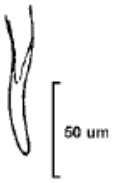
(4a)

(4b)

5. a. Sender larva, tail of sheath medium length (> 50 um < 100 um), tapering to point, often kinked ..... ***Haemonchus***
   b. Tail of sheath very short (< 50 um), conical **6**

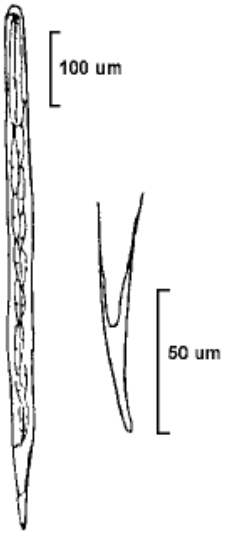


(5a)

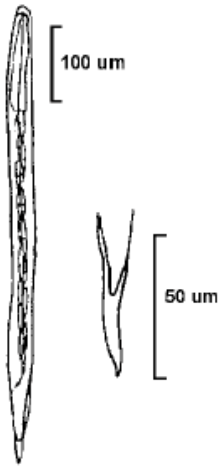


(5b)

6. a. Larva of medium or large size with distinct rounded tail in short sheath ..... ***Ostertagia***
   b. Small larva, tail being indistinctly rounded ..... ***Trichostrongylus***



(6a)



(6b)

Fig. A.1 Continued.

7. a. Very large larva, 8 gut cells, tail notched, bilobed or trilobed ..... ***Nematodirus***
   b. Larva of medium size, 32 pentagonal gut cells, lumen of gut wavy ..... ***Oesophagostomum***
   c. Larva medium size 32 square gut cells, lumen of gut straight ..... ***Chabertia***
   d. Very small larva with 16 gut cells ..... ***Bunostomum***

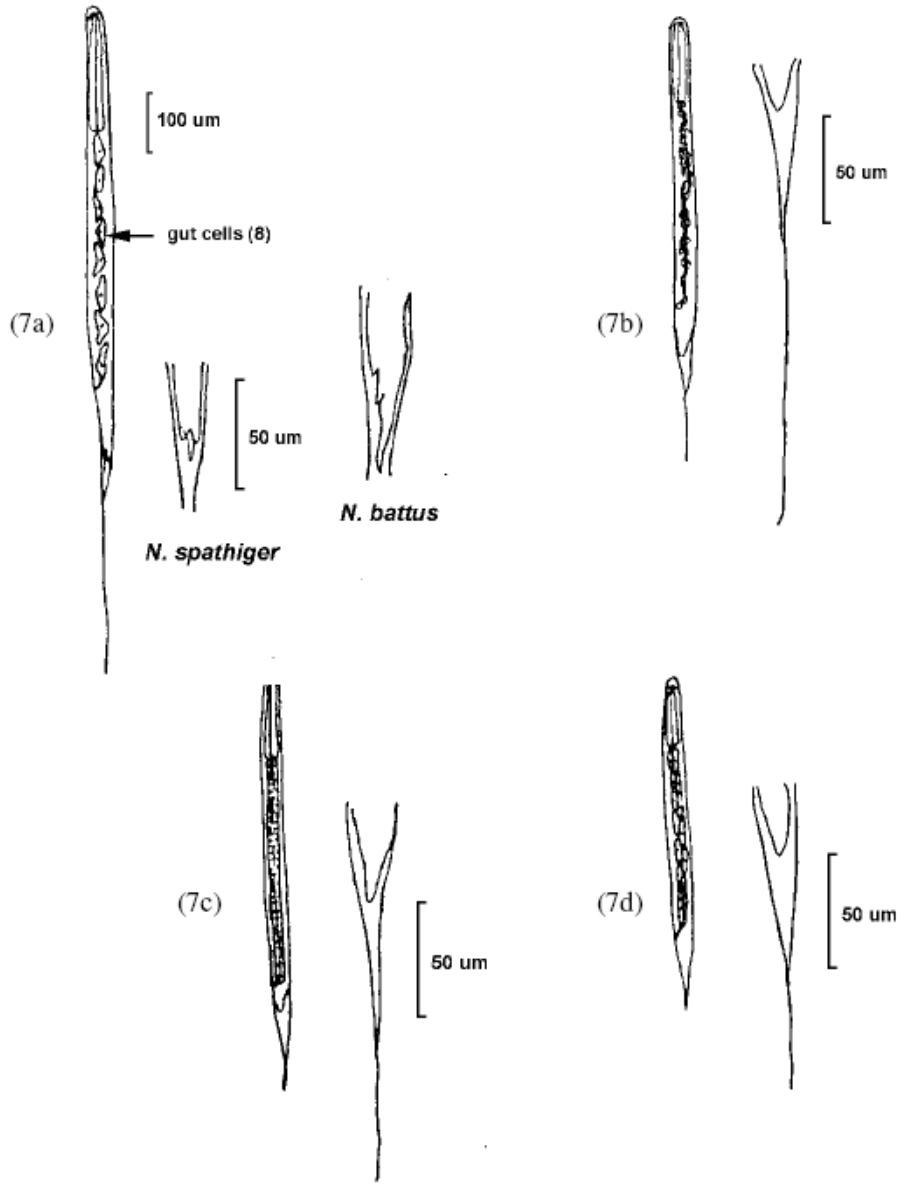


Fig. A.1 Continued.

APPENDIX B

Table B.1
DrenchRite® assay counts for Hc-ES. ** Final egg count included in % L_1/L_2.

Hc-ES	Wells											
BZ - A	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	20	21	13	0	21	27	25	15	17	26	20	30
L_1 / L_2	4	3	2	0	11	15	14	5	4	2	0	0
L_3 Dead	1	1	0	0	2	1	0	0	0	0	0	0
L_3 Alive	10	10	6	0	3	3	2	0	0	0	0	0
Total Larvae	15	14	8	0	16	19	16	5	4	2	0	0
Final egg	5	7	5	0	5	8	9	10	13	24	20	30
% L_1 / L_2	27	21	25	N/A	76	85	92	100	100	100	100	100
% L_3	73	79	75	N/A	24	15	8	0	0	0	0	0
BZ - B	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	14	26	22	18	24	20	23	27	21	22	29	32
L_1 / L_2	0	2	2	3	10	10	7	0	5	2	0	0
L_3 Dead	2	0	0	0	1	3	3	2	0	0	0	0
L_3 Alive	9	16	12	9	8	3	1	0	0	0	0	0
Total Larvae	11	18	14	12	19	16	11	2	5	2	0	0
Final egg	3	8	8	6	5	4	12	25	16	20	29	32
% L_1 / L_2	0	11	14	25	63	70	83	93	100	100	100	100
% L_3	100	89	86	75	37	30	17	7	0	0	0	0
LEV - C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	23	17	25	15	27	19	18	21	19	16	22	23
L_1 / L_2	5	5	6	2	1	3	3	6	7	7	17	16
L_3 Dead	0	1	1	0	1	0	1	1	0	1	0	1
L_3 Alive	14	4	14	7	14	8	7	4	9	4	1	2
Total Larvae	19	10	21	9	16	11	11	11	16	12	18	19
Final egg	4	7	4	6	11	8	7	10	3	4	4	4
% L_1 / L_2	26	50	29	22	6	27	27	55	44	58	94	84
% L_3	74	50	71	78	94	73	73	45	56	42	6	16
LEV - D	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	23	19	15	18	10	25	18	17	21	19	17	20
L_1 / L_2	2	1	2	8	1	11	7	8	8	6	7	8
L_3 Dead	0	0	1	0	1	1	1	0	1	2	0	2
L_3 Alive	13	8	8	4	6	7	4	3	3	2	4	3
Total Larvae	15	9	11	12	8	19	12	11	12	10	11	13
Final egg	8	10	4	6	2	6	6	6	9	9	6	7
% L_1 / L_2	13	11	18	67	12	58	58	73	67	60	64	62
% L_3	87	89	82	33	88	42	42	27	33	40	36	38

Table B.1 Continued

Hc-ES	Wells											
BZ/LEV - E	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	19	15	23	25	17	21	24	20	21	22	26	20
L_1 / L_2	4	2	3	10	10	13	18	15	14	14	18	10
L_3 Dead	0	0	1	3	0	2	0	0	0	0	0	0
L_3 Alive	7	8	13	7	2	0	0	0	0	0	0	0
Total Larvae	11	10	17	20	12	15	18	15	14	14	18	10
Final egg	8	5	6	5	5	6	6	5	7	8	8	10
% L_1 / L_2	36	20	18	50	83	87	100	100	100	100	100	100
% L_3	64	80	82	50	17	13	0	0	0	0	0	0
BZ/LEV - F	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	19	18	20	14	20	20	17	24	24	14	18	18
L_1 / L_2	0	1	3	4	8	14	11	16	15	9	13	10
L_3 Dead	1	0	0	2	3	0	0	0	0	0	0	0
L_3 Alive	11	8	7	3	1	0	0	0	0	0	0	0
Total Larvae	12	9	10	9	12	14	11	16	15	9	13	10
Final egg	7	9	10	5	8	6	6	8	9	5	5	8
% L_1 / L_2	0	11	30	44	67	100	100	100	100	100	100	100
% L_3	100	89	70	56	33	0	0	0	0	0	0	0
AVM - G	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	23	17	15	19	13	20	15	22	26	22	18	25
L_1 / L_2	4	3	7	7	6	14	9	13	21	19	12	18
L_3 Dead	1	1	0	1	0	0	0	0	0	0	0	0
L_3 Alive	13	6	4	0	0	0	0	0	0	0	0	0
Total Larvae	18	10	11	8	6	14	9	13	21	19	12	18
Final egg	5	7	4	11	7	6	6	9	5	3	6	7
% L_1 / L_2	22	30	64	88	100	100	100	100	100	100	100	100
% L_3	78	70	36	12	0	0	0	0	0	0	0	0
AVM - H	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	27	17	15	21	22	21	24	20	15	20	32	20
L_1 / L_2	10	5	4	15	17	18	19	15	11	14	27	13
L_3 Dead	1	0	1	0	0	0	0	0	0	0	0	0
L_3 Alive	11	5	3	3	0	0	0	0	0	0	0	0
Total Larvae	22	10	8	18	17	18	19	15	11	14	27	13
Final egg	5	7	7	3	5	3	5	5	4	6	5	7
% L_1 / L_2	45	50	50	83	100	100	100	100	100	100	100	100
% L_3	55	50	50	17	0	0	0	0	0	0	0	0

Table B.2
DrenchRite® assay averages for Hc-ES. ** Final egg count included in % L_1/L_2.

Hc-ES	Wells											
BZ AVG	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	34	47	35	18	45	47	48	42	38	48	49	62
L_1 / L_2	4	5	4	3	21	25	21	5	9	4	0	0
L_3 Dead	3	1	0	0	3	4	3	2	0	0	0	0
L_3 Alive	19	26	18	9	11	6	3	0	0	0	0	0
Total Larvae	26	32	22	12	35	35	27	7	9	4	0	0
Final egg	8	15	13	6	10	12	21	35	29	44	49	62
% L_1 / L_2	15	16	18	25	69	79	88	95	100	100	100	100
% L_3	85	84	82	75	31	21	12	5	0	0	0	0
LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	46	36	40	33	37	44	36	38	40	35	39	43
L_1 / L_2	7	6	8	10	2	14	10	14	15	13	24	24
L_3 Dead	0	1	2	0	2	1	2	1	1	3	0	3
L_3 Alive	27	12	22	11	20	15	11	7	12	6	5	5
Total Larvae	34	19	32	21	24	30	23	22	28	22	29	32
Final egg	12	17	8	12	13	14	13	16	12	13	10	11
% L_1 / L_2	21	32	25	48	8	47	43	64	54	59	83	75
% L_3	79	68	75	52	92	53	57	36	46	41	17	25
BZ/LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	38	33	43	39	37	41	41	44	45	36	44	38
L_1 / L_2	4	3	6	14	18	27	29	31	29	23	31	20
L_3 Dead	1	0	1	5	3	2	0	0	0	0	0	0
L_3 Alive	18	16	20	10	3	0	0	0	0	0	0	0
Total Larvae	23	19	27	29	24	29	29	31	29	23	31	20
Final egg	15	14	16	10	13	12	12	13	16	13	13	18
% L_1 / L_2	17	16	22	48	75	93	100	100	100	100	100	100
% L_3	83	84	78	52	25	7	0	0	0	0	0	0
AVM AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	50	34	30	40	35	41	39	42	41	42	50	45
L_1 / L_2	14	8	11	22	23	32	28	28	32	33	39	31
L_3 Dead	2	1	1	1	0	0	0	0	0	0	0	0
L_3 Alive	24	11	7	3	0	0	0	0	0	0	0	0
Total Larvae	40	20	19	26	23	32	28	28	32	33	39	31
Final egg	10	14	11	14	12	9	11	14	9	9	11	14
% L_1 / L_2	35	40	58	85	100	100	100	100	100	100	100	100
% L_3	65	60	42	15	0	0	0	0	0	0	0	0

Table B.3
DrenchRite® assay counts for Hc-EG. ** Final egg count included in % L_1/L_2.

Hc-EG	Wells											
BZ - A	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	34	32	32	44	30	26	42	35	36	41	35	47
L_1 / L_2	1	3	5	8	3	12	20	14	14	8	11	3
L_3 Dead	1	1	1	1	3	2	4	4	1	2	2	0
L_3 Alive	29	26	19	25	16	9	10	5	6	8	1	0
Total Larvae	31	30	25	34	22	23	34	23	21	18	14	3
Final egg	3	2	7	10	8	3	8	12	15	23	21	44
% L_1 / L_2	3	10	20	24	37	58	67	74	81	76	91	100
% L_3	97	90	80	76	63	42	33	26	19	24	9	0
BZ - B	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	28	22	39	34	21	37	42	36	38	42	24	32
L_1 / L_2	5	2	8	6	5	16	16	15	12	13	2	0
L_3 Dead	2	0	2	0	1	3	6	5	4	4	1	1
L_3 Alive	14	12	23	16	14	13	14	8	3	6	2	0
Total Larvae	21	14	33	22	20	32	36	28	19	23	5	1
Final egg	7	8	6	12	1	5	6	8	19	19	19	31
% L_1 / L_2	24	14	24	27	29	57	52	64	82	76	88	97
% L_3	76	86	76	73	71	43	48	36	18	24	12	3
LEV - C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	31	37	36	27	30	26	49	34	43	32	52	35
L_1 / L_2	4	1	3	2	4	3	8	12	20	18	35	28
L_3 Dead	1	0	1	0	2	2	4	1	2	2	3	2
L_3 Alive	20	32	24	19	17	17	28	12	10	7	7	4
Total Larvae	25	33	28	21	23	22	40	25	32	27	45	34
Final egg	6	4	8	6	7	4	9	9	11	5	7	1
% L_1 / L_2	16	3	11	10	17	14	20	48	63	67	78	82
% L_3	84	97	89	90	83	86	80	52	73	33	22	18
LEV - D	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	18	31	24	47	43	34	37	34	46	38	42	31
L_1 / L_2	1	0	5	2	2	2	7	11	28	24	23	22
L_3 Dead	0	1	0	1	0	0	2	2	3	1	1	0
L_3 Alive	13	26	15	31	32	27	23	16	4	6	5	3
Total Larvae	14	27	20	34	34	29	32	29	35	31	29	25
Final egg	4	4	4	13	9	5	5	5	11	7	13	6
% L_1 / L_2	7	0	25	6	6	7	22	38	80	77	79	88
% L_3	93	100	75	94	94	93	78	62	20	23	21	12

Table B.3 Continued

Hc-EG	Wells											
BZ/LEV - E	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	37	19	37	38	38	34	46	49	55	45	44	34
L_1 / L_2	1	0	4	11	17	20	34	43	45	32	35	24
L_3 Dead	0	2	0	2	2	2	4	1	0	0	0	0
L_3 Alive	27	14	28	20	12	2	1	1	0	0	0	0
Total Larvae	28	16	32	33	31	24	39	45	45	32	35	24
Final egg	9	3	5	5	7	10	7	4	10	13	9	10
% L_1 / L_2	4	0	13	33	55	83	87	96	100	100	100	100
% L_3	96	100	87	67	45	17	13	4	0	0	0	0
BZ/LEV - F	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	30	40	39	24	36	35	30	84	35	33	42	43
L_1 / L_2	2	2	9	9	16	18	23	76	25	27	28	33
L_3 Dead	0	0	1	0	3	2	1	0	0	0	0	0
L_3 Alive	15	33	28	12	10	5	2	0	0	0	0	0
Total Larvae	17	35	38	21	29	25	26	76	25	27	28	33
Final egg	13	5	1	3	7	10	4	8	10	6	14	10
% L_1 / L_2	12	6	24	43	55	72	88	100	100	100	100	100
% L_3	88	94	76	57	45	28	12	0	0	0	0	0
AVM - G	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	27	38	37	36	37	39	31	73	43	30	35	40
L_1 / L_2	3	3	9	6	17	15	21	50	35	28	27	32
L_3 Dead	2	0	0	0	1	1	0	2	0	0	0	0
L_3 Alive	15	26	21	23	15	14	2	9	1	0	0	0
Total Larvae	20	29	30	29	33	30	23	61	36	28	27	32
Final egg	7	9	7	7	4	9	8	12	7	2	8	8
% L_1 / L_2	15	10	30	21	52	50	91	82	97	100	100	100
% L_3	85	90	70	79	48	50	9	18	3	0	0	0
AVM - H	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	32	30	41	29	32	46	46	74	35	33	34	36
L_1 / L_2	1	7	6	5	10	21	29	47	27	25	26	29
L_3 Dead	1	0	1	0	0	0	0	3	1	0	0	0
L_3 Alive	25	16	23	13	12	14	9	7	2	1	1	0
Total Larvae	27	23	30	18	22	35	38	57	30	26	27	29
Final egg	5	7	11	11	10	11	8	17	5	7	7	7
% L_1 / L_2	4	30	20	28	45	60	76	82	90	96	96	100
% L_3	96	70	80	72	55	40	24	18	10	4	4	0

Table B.4
DrenchRite® assay averages for Hc-EG. ** Final egg count included in % L_1/L_2.

Hc-EG	Wells											
BZ AVG	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	62	54	71	78	51	63	84	71	74	83	59	79
L_1 / L_2	6	5	13	14	8	28	36	29	26	21	13	3
L_3 Dead	3	1	3	1	4	5	10	9	5	6	3	1
L_3 Alive	43	38	42	41	30	22	24	13	9	14	3	0
Total Larvae	52	44	58	56	42	55	70	51	40	41	19	4
Final egg	10	10	13	22	9	8	14	20	34	42	40	75
% L_1 / L_2	12	11	22	25	33	57	60	69	81	76	90	99
% L_3	88	89	78	75	67	43	40	31	19	24	10	1
LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	49	68	60	74	73	60	86	68	89	70	94	66
L_1 / L_2	5	1	8	4	6	5	15	23	48	42	58	50
L_3 Dead	1	1	1	1	2	2	6	3	5	3	4	2
L_3 Alive	33	58	39	50	49	44	51	28	14	13	12	7
Total Larvae	39	60	48	55	57	51	72	54	67	58	74	59
Final egg	10	8	12	19	16	9	14	14	22	12	20	7
% L_1 / L_2	13	2	17	7	11	10	21	43	72	72	78	85
% L_3	87	98	83	93	89	90	79	57	28	28	22	15
BZ/LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	67	59	76	62	74	69	76	133	90	78	86	77
L_1 / L_2	3	2	13	20	33	38	57	119	70	59	63	57
L_3 Dead	0	2	1	2	5	4	5	1	0	0	0	0
L_3 Alive	42	47	56	32	22	7	3	1	0	0	0	0
Total Larvae	45	51	70	54	60	49	65	121	70	59	63	57
Final egg	22	8	6	8	14	20	11	12	20	19	23	20
% L_1 / L_2	7	4	19	37	55	78	88	98	100	100	100	100
% L_3	93	96	81	63	45	22	12	2	0	0	0	0
AVM AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	59	68	78	65	69	85	77	147	78	63	69	76
L_1 / L_2	4	10	15	11	27	36	50	97	62	53	53	61
L_3 Dead	3	0	1	0	1	1	0	5	1	0	0	0
L_3 Alive	40	42	44	36	27	28	11	16	3	1	1	0
Total Larvae	47	52	60	47	55	65	61	118	66	54	54	61
Final egg	12	16	18	18	14	20	16	29	12	9	15	15
% L_1 / L_2	9	19	25	23	49	55	82	82	94	98	98	100
% L_3	91	81	75	77	51	45	18	18	6	2	2	0

Table B.5
DrenchRite® assay counts for Hc-OS. ** Final egg count included in % L_1/L_2.

Hc-OS	Wells											
BZ - A	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	45	20	29	25	26	20	30	25	65	27	36	18
L_1 / L_2	3	1	3	6	11	14	18	5	10	4	1	1
L_3 Dead	1	2	1	0	1	1	0	1	0	0	1	1
L_3 Alive	30	15	15	16	9	4	4	0	0	0	0	0
Total Larvae	34	18	19	22	21	19	22	6	10	4	2	2
Final egg	11	2	10	3	5	1	8	19	55	23	34	16
% L_1 / L_2	9	6	16	27	62	75	87	96	100	100	97	94
% L_3	91	94	84	73	38	25	13	4	0	0	3	6
BZ - B	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	30	29	32	33	42	46	36	37	48	44	32	45
L_1 / L_2	4	2	3	4	23	25	21	14	9	3	1	3
L_3 Dead	1	1	0	2	3	2	0	2	2	1	0	2
L_3 Alive	20	18	24	16	13	7	3	0	0	0	0	0
Total Larvae	25	21	27	22	39	34	24	16	11	4	1	5
Final egg	5	8	5	11	3	12	12	21	37	40	31	40
% L_1 / L_2	16	10	11	18	62	80	92	95	96	98	100	96
% L_3	84	90	89	82	38	20	8	5	4	2	0	4
LEV - C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	54	34	39	26	88	37	48	41	38	39	37	41
L_1 / L_2	6	6	8	6	29	16	27	29	37	27	28	32
L_3 Dead	0	0	0	1	5	3	0	0	0	4	2	0
L_3 Alive	31	21	17	16	36	5	10	6	0	0	1	1
Total Larvae	37	27	25	23	70	24	37	35	37	31	31	33
Final egg	17	7	14	3	18	13	11	6	1	8	6	8
% L_1 / L_2	16	22	32	26	41	67	73	83	100	87	90	97
% L_3	84	78	68	74	59	33	27	17	0	13	10	3
LEV - D	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	34	31	36	24	37	46	54	31	26	34	36	49
L_1 / L_2	2	2	3	3	12	26	33	20	24	32	27	37
L_3 Dead	0	1	1	1	1	3	1	0	0	0	0	1
L_3 Alive	23	22	28	19	22	5	4	3	0	0	1	1
Total Larvae	25	25	32	23	35	34	38	23	24	32	28	39
Final egg	9	6	4	1	2	12	16	8	2	2	8	10
% L_1 / L_2	8	8	9	13	34	76	87	87	100	100	96	95
% L_3	92	92	91	87	66	24	13	13	0	0	4	5

Table B.5 Continued

Hc-OS	Wells											
BZ/LEV - E	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	46	47	36	51	38	32	43	39	44	45	46	35
L_1 / L_2	6	4	4	16	24	25	41	37	36	38	44	28
L_3 Dead	0	0	0	0	0	2	0	0	0	0	0	0
L_3 Alive	29	32	22	19	10	1	0	0	0	0	0	0
Total Larvae	35	36	26	35	34	28	41	37	36	38	44	28
Final egg	11	11	10	16	4	4	2	2	8	7	2	7
% L_1 / L_2	17	11	15	46	71	89	100	100	100	100	100	100
% L_3	83	89	85	54	29	11	0	0	0	0	0	0
BZ/LEV - F	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	50	76	47	46	44	52	41	47	58	57	61	49
L_1 / L_2	5	5	3	11	30	43	39	45	53	43	60	46
L_3 Dead	0	2	1	3	0	0	0	0	0	0	0	0
L_3 Alive	34	47	35	24	8	2	0	0	0	0	0	0
Total Larvae	39	54	39	38	38	45	39	45	53	43	60	46
Final egg	11	22	8	8	6	7	2	2	5	14	1	3
% L_1 / L_2	13	9	8	29	79	96	100	100	100	100	100	100
% L_3	87	91	92	71	21	4	0	0	0	0	0	0
AVM - G	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	51	62	64	42	73	63	46	37	54	59	56	69
L_1 / L_2	4	8	22	29	56	46	40	33	48	46	54	55
L_3 Dead	0	1	2	1	0	0	0	0	0	0	0	0
L_3 Alive	31	39	22	9	7	6	1	0	0	0	0	0
Total Larvae	35	48	46	39	63	52	41	33	48	46	54	55
Final egg	16	14	18	3	10	11	5	4	6	13	2	14
% L_1 / L_2	11	17	48	74	89	88	98	100	100	100	100	100
% L_3	89	83	52	26	11	12	2	0	0	0	0	0
AVM - H	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	38	36	84	23	78	63	42	38	67	57	48	41
L_1 / L_2	5	4	38	13	53	49	41	33	62	51	47	37
L_3 Dead	0	1	2	0	0	0	0	1	1	0	0	0
L_3 Alive	15	17	25	8	10	2	1	1	0	0	0	0
Total Larvae	20	22	65	21	63	51	42	35	63	51	47	37
Final egg	18	14	19	2	15	12	0	3	4	6	1	4
% L_1 / L_2	25	18	58	62	84	96	98	94	98	100	100	100
% L_3	75	82	42	38	16	4	2	6	2	0	0	0

Table B.6
DrenchRite® assay averages for Hc-OS. ** Final egg count included in % L_1/L_2.

Hc-OS	Wells											
BZ AVG	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	75	49	61	58	68	66	66	62	113	71	68	63
L_1 / L_2	7	3	6	10	34	39	39	19	19	7	2	4
L_3 Dead	2	3	1	2	4	3	0	3	2	1	1	3
L_3 Alive	50	33	39	32	22	11	7	0	0	0	0	0
Total Larvae	59	39	46	44	60	53	46	22	21	8	3	7
Final egg	16	10	15	14	8	13	20	40	92	63	65	56
% L_1 / L_2	12	8	13	23	62	79	89	95	98	99	99	95
% L_3	88	92	87	77	38	21	11	5	2	1	1	5
LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	88	65	75	50	125	83	102	72	64	73	73	90
L_1 / L_2	8	8	11	9	41	42	60	49	61	59	55	69
L_3 Dead	0	1	1	2	6	6	1	0	0	4	2	1
L_3 Alive	54	43	45	35	58	10	14	9	0	0	2	2
Total Larvae	62	52	57	46	105	58	75	58	61	63	59	72
Final egg	26	13	18	4	20	25	27	14	3	10	14	18
% L_1 / L_2	13	15	19	20	39	72	80	84	100	94	93	96
% L_3	87	85	81	80	61	28	20	16	0	6	7	4
BZ/LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	96	123	83	97	82	84	84	86	102	102	107	84
L_1 / L_2	11	9	7	27	54	68	80	82	89	81	104	74
L_3 Dead	0	2	1	3	0	2	0	0	0	0	0	0
L_3 Alive	63	79	57	43	18	3	0	0	0	0	0	0
Total Larvae	74	90	65	73	72	73	80	82	89	81	104	74
Final egg	22	33	18	24	10	11	4	4	13	21	3	10
% L_1 / L_2	15	10	11	37	75	93	100	100	100	100	100	100
% L_3	85	90	89	63	25	7	0	0	0	0	0	0
AVM AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	89	98	148	65	151	126	88	75	121	116	104	110
L_1 / L_2	9	12	60	42	109	95	81	66	110	97	101	92
L_3 Dead	0	2	4	1	0	0	0	1	1	0	0	0
L_3 Alive	46	56	47	17	17	8	2	1	0	0	0	0
Total Larvae	55	70	111	60	126	103	83	68	111	97	101	92
Final egg	34	28	37	5	25	23	5	7	10	19	3	18
% L_1 / L_2	16	17	54	70	87	92	98	97	99	100	100	100
% L_3	84	83	46	30	13	8	2	3	1	0	0	0

Table B.7
DrenchRite® assay counts for Hc-OG. ** Final egg count included in % L_1/L_2.

Hc-OG	Wells											
BZ - A	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	59	44	32	37	41	46	37	51	41	46	43	32
L_1 / L_2	7	7	2	4	2	12	12	11	10	3	2	1
L_3 Dead	0	0	0	0	0	2	1	2	5	2	0	0
L_3 Alive	13	14	15	12	8	7	3	9	3	0	0	0
Total Larvae	20	21	17	16	10	21	16	22	18	5	2	1
Final egg	39	23	15	21	31	25	21	29	23	41	41	31
% L_1 / L_2	35	33	12	25	80	80	89	78	80	96	100	100
% L_3	65	67	88	75	20	20	11	22	20	4	0	0
BZ - B	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	62	59	45	36	45	49	55	48	50	64	48	38
L_1 / L_2	12	4	8	5	7	15	14	22	13	17	3	2
L_3 Dead	0	3	0	0	1	2	2	3	3	2	1	0
L_3 Alive	21	23	13	10	13	12	10	2	3	0	1	0
Total Larvae	33	30	21	15	21	29	26	27	19	19	5	2
Final egg	29	29	24	21	24	20	29	21	31	45	43	36
% L_1 / L_2	36	13	38	33	69	71	78	90	88	97	96	100
% L_3	64	87	62	67	31	29	22	10	12	3	4	0
LEV - C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	57	66	67	23	32	53	77	49	42	61	59	45
L_1 / L_2	6	7	3	14	11	21	34	23	14	28	31	20
L_3 Dead	0	0	0	0	1	3	2	3	3	2	1	1
L_3 Alive	16	22	27	7	6	2	2	2	1	0	0	1
Total Larvae	22	29	30	21	18	26	38	28	18	30	32	22
Final egg	35	37	37	2	14	27	39	21	24	31	27	23
% L_1 / L_2	27	24	10	67	61	81	89	82	78	93	97	91
% L_3	73	76	90	33	39	19	11	18	22	7	3	9
LEV - D	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	69	40	49	44	55	63	48	53	41	63	52	49
L_1 / L_2	5	6	7	8	10	20	24	22	19	19	27	15
L_3 Dead	2	2	0	0	3	5	2	1	1	2	1	1
L_3 Alive	9	9	14	16	11	4	1	0	2	1	0	0
Total Larvae	16	17	21	24	24	29	27	23	22	22	28	16
Final egg	53	23	28	20	31	34	21	30	19	41	24	33
% L_1 / L_2	31	35	33	33	42	69	89	96	86	86	96	94
% L_3	69	65	67	67	58	31	11	4	14	14	4	6

Table B.7 Continued

Hc-OG	Wells											
BZ/LEV - E	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	65	61	47	60	55	59	50	63	49	57	46	51
L_1 / L_2	8	7	8	7	10	27	23	29	21	26	26	21
L_3 Dead	2	0	2	2	2	2	0	0	0	0	0	0
L_3 Alive	28	24	11	18	11	1	0	0	0	0	0	0
Total Larvae	38	31	21	27	23	30	23	29	21	26	26	21
Final egg	27	30	26	33	32	29	27	34	28	31	20	30
% L_1 / L_2	21	23	38	26	43	90	100	100	100	100	100	100
% L_3	79	77	62	74	57	10	0	0	0	0	0	0
BZ/LEV - F	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	61	75	60	57	64	52	45	53	53	44	57	53
L_1 / L_2	6	6	9	10	20	19	26	26	15	11	23	28
L_3 Dead	1	0	2	2	3	1	0	0	0	0	0	0
L_3 Alive	29	18	16	14	12	2	0	0	0	0	0	0
Total Larvae	36	24	27	26	35	22	26	26	15	11	23	28
Final egg	25	51	33	31	29	30	19	27	38	33	34	25
% L_1 / L_2	17	25	33	38	57	86	100	100	100	100	100	100
% L_3	83	75	67	62	43	14	0	0	0	0	0	0
AVM - G	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	54	70	57	50	51	62	57	54	45	58	49	52
L_1 / L_2	2	12	13	22	25	31	31	32	24	28	27	28
L_3 Dead	0	0	0	3	0	1	1	0	0	0	0	0
L_3 Alive	18	21	13	7	2	2	0	0	0	0	0	0
Total Larvae	20	33	26	32	27	34	32	32	24	28	27	28
Final egg	34	37	31	18	24	28	25	22	21	30	22	24
% L_1 / L_2	10	36	50	69	93	91	97	100	100	100	100	100
% L_3	90	64	50	31	7	9	3	0	0	0	0	0
AVM - H	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	42	49	60	53	34	61	50	44	48	42	72	72
L_1 / L_2	6	11	10	22	17	34	24	13	17	14	31	31
L_3 Dead	0	2	2	1	3	0	1	0	0	0	0	0
L_3 Alive	12	15	22	4	1	0	0	0	1	0	0	0
Total Larvae	18	28	34	27	21	34	25	13	18	14	31	31
Final egg	24	21	26	26	13	27	25	31	30	28	41	41
% L_1 / L_2	33	39	29	81	81	100	96	100	94	100	100	100
% L_3	67	61	71	19	19	0	4	0	6	0	0	0

Table B.8
DrenchRite® assay averages for Hc-OG. ** Final egg count included in % L_1/L_2.

Hc-OG	Wells											
BZ AVG	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	121	103	77	73	86	95	92	99	91	110	91	70
L_1 / L_2	19	11	10	9	9	27	26	33	23	20	5	3
L_3 Dead	0	3	0	0	1	4	3	5	8	4	1	0
L_3 Alive	34	37	28	22	21	19	13	11	6	0	1	0
Total Larvae	53	51	38	31	31	50	42	49	37	24	7	3
Final egg	68	52	39	42	55	45	50	50	54	86	84	67
% L_1 / L_2	36	22	26	29	74	76	83	84	85	96	98	100
% L_3	64	78	74	71	26	24	17	16	15	4	2	0
LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	126	106	116	67	87	116	125	102	83	124	111	94
L_1 / L_2	11	13	10	22	21	41	58	45	33	47	58	35
L_3 Dead	2	2	0	0	4	8	4	4	4	4	2	2
L_3 Alive	25	31	41	23	17	6	3	2	3	1	0	1
Total Larvae	38	46	51	45	42	55	65	51	40	52	60	38
Final egg	88	60	65	22	45	61	60	51	43	72	51	56
% L_1 / L_2	29	28	20	49	50	75	89	88	83	90	97	92
% L_3	71	72	80	51	50	25	11	12	17	10	3	8
BZ/LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	126	136	107	117	119	111	95	116	102	101	103	104
L_1 / L_2	14	13	17	17	30	46	49	55	36	37	49	49
L_3 Dead	3	0	4	4	5	3	0	0	0	0	0	0
L_3 Alive	57	42	27	32	23	3	0	0	0	0	0	0
Total Larvae	74	55	48	53	58	52	49	55	36	37	49	49
Final egg	52	81	59	64	61	59	46	61	66	64	54	55
% L_1 / L_2	19	24	35	32	52	88	100	100	100	100	100	100
% L_3	81	76	65	68	48	12	0	0	0	0	0	0
AVM AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	96	119	117	103	85	123	107	98	93	100	121	124
L_1 / L_2	8	23	23	44	42	65	55	45	41	42	58	59
L_3 Dead	0	2	2	4	3	1	2	0	0	0	0	0
L_3 Alive	30	36	35	11	3	2	0	0	1	0	0	0
Total Larvae	38	61	60	59	48	68	57	45	42	42	58	59
Final egg	58	58	57	44	37	55	50	53	51	58	63	65
% L_1 / L_2	21	38	38	75	88	96	96	100	98	100	100	100
% L_3	79	62	62	25	12	4	4	0	2	0	0	0

Table B.9
DrenchRite® assay counts for Hc-RFR. ** Final egg count included in % L_1/L_2.

Hc-RFR	Wells											
BZ - A	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	54	36	33	50	53	47	35	53	47	44	37	34
L_1 / L_2	9	3	2	5	6	2	3	6	2	10	7	5
L_3 Dead	2	1	0	0	2	0	1	2	1	1	2	2
L_3 Alive	21	19	21	29	27	30	13	26	28	12	8	0
Total Larvae	32	23	23	34	35	32	17	34	31	23	17	7
Final egg	22	13	10	16	18	15	18	19	16	21	20	27
% L_1 / L_2	28	13	9	15	45	36	60	47	38	70	73	94
% L_3	72	87	91	85	55	64	40	53	62	30	27	6
BZ - B	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	61	42	47	42	40	46	56	68	58	47	56	43
L_1 / L_2	6	3	0	1	4	2	3	7	4	15	17	8
L_3 Dead	6	2	0	3	1	1	3	4	0	2	2	1
L_3 Alive	25	24	29	24	19	22	29	27	28	18	9	2
Total Larvae	37	29	29	28	24	25	35	38	32	35	28	11
Final egg	24	13	18	14	16	21	21	30	26	12	28	32
% L_1 / L_2	16	10	0	4	50	50	43	54	52	57	80	93
% L_3	84	90	100	96	50	50	57	46	48	43	20	7
LEV - C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	45	49	38	56	54	44	37	42	51	47	45	43
L_1 / L_2	3	1	2	12	22	16	17	29	29	40	28	31
L_3 Dead	0	0	1	1	2	1	1	3	1	0	1	0
L_3 Alive	27	29	26	23	17	5	3	1	0	0	1	1
Total Larvae	30	30	29	36	41	22	21	33	30	40	30	32
Final egg	15	19	9	20	13	22	16	9	21	7	15	11
% L_1 / L_2	10	3	7	33	54	73	81	88	97	100	93	97
% L_3	90	97	93	67	46	27	19	12	3	0	7	3
LEV - D	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	53	35	42	45	43	44	44	37	42	46	41	37
L_1 / L_2	4	0	2	8	11	19	27	28	21	31	22	29
L_3 Dead	1	2	1	1	4	0	1	1	0	0	0	0
L_3 Alive	29	21	28	23	16	2	1	2	1	0	0	0
Total Larvae	34	23	31	32	31	21	29	31	22	31	22	29
Final egg	19	12	11	13	12	23	15	6	20	15	19	8
% L_1 / L_2	12	0	6	25	35	90	93	90	95	100	100	100
% L_3	88	100	94	75	65	10	7	10	5	0	0	0

Table B.9 Continued

Hc-RFR	Wells											
BZ/LEV - E	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	64	47	55	46	46	44	35	40	58	53	35	40
L_1 / L_2	6	7	5	4	6	10	23	22	40	23	22	24
L_3 Dead	2	3	1	1	1	2	0	2	0	0	0	0
L_3 Alive	32	24	33	26	22	10	2	2	0	0	0	0
Total Larvae	40	34	39	31	29	22	25	26	40	23	22	24
Final egg	24	13	16	15	17	22	10	14	18	30	13	16
% L_1 / L_2	15	21	13	13	21	45	92	85	100	100	100	100
% L_3	85	79	87	87	79	55	8	15	0	0	0	0
BZ/LEV - F	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	51	54	46	53	36	51	52	38	53	41	46	44
L_1 / L_2	4	4	4	3	5	20	36	23	37	25	30	29
L_3 Dead	0	1	1	3	1	1	1	1	0	0	0	0
L_3 Alive	33	37	29	24	17	9	4	3	0	0	0	0
Total Larvae	37	42	34	30	23	30	41	27	37	25	30	29
Final egg	14	12	12	23	13	21	11	11	16	16	16	15
% L_1 / L_2	11	10	12	10	22	67	88	85	100	100	100	100
% L_3	89	90	88	90	78	33	12	15	0	0	0	0
AVM - G	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	71	81	59	38	49	57	56	37	88	46	73	53
L_1 / L_2	3	9	6	7	7	6	33	19	39	25	38	27
L_3 Dead	0	0	0	0	0	1	2	0	0	1	0	0
L_3 Alive	39	44	40	22	24	22	1	4	1	1	0	0
Total Larvae	42	53	46	29	31	29	36	23	40	27	38	27
Final egg	29	28	13	9	18	28	20	14	48	19	35	26
% L_1 / L_2	7	17	13	24	23	21	92	83	98	93	100	100
% L_3	93	83	87	76	77	79	8	17	3	7	0	0
AVM - H	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	66	41	40	39	49	49	53	37	45	55	52	52
L_1 / L_2	3	7	4	3	6	13	24	23	29	33	29	33
L_3 Dead	3	1	1	0	1	0	0	0	0	0	0	0
L_3 Alive	37	25	23	21	23	14	7	5	1	0	0	0
Total Larvae	43	33	28	24	30	27	31	28	30	33	29	33
Final egg	23	8	12	15	19	22	22	9	15	22	23	19
% L_1 / L_2	7	21	14	13	20	48	77	82	97	100	100	100
% L_3	93	79	86	87	80	52	23	18	3	0	0	0

Table B.10
DrenchRite® assay averages for Hc-RFR. ** Final egg count included in %L_1/L_2.

Hc-RFR	Wells											
BZ AVG	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	115	78	80	92	93	93	91	121	105	91	93	77
L_1 / L_2	15	6	2	6	10	4	6	13	6	25	24	13
L_3 Dead	8	3	0	3	3	1	4	6	1	3	4	3
L_3 Alive	46	43	50	53	46	52	42	53	56	30	17	2
Total Larvae	69	52	52	62	59	57	52	72	63	58	45	18
Final egg	46	26	28	30	34	36	39	49	42	33	48	59
% L_1 / L_2	22	12	4	10	47	43	49	51	46	64	77	94
% L_3	78	88	96	90	53	57	51	49	54	36	23	6
LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	98	84	80	101	97	88	81	79	93	93	86	80
L_1 / L_2	7	1	4	20	33	35	44	57	50	71	50	60
L_3 Dead	1	2	2	2	6	1	2	4	1	0	1	0
L_3 Alive	56	50	54	46	33	7	4	3	1	0	1	1
Total Larvae	64	53	60	68	72	43	50	64	52	71	52	61
Final egg	34	31	20	33	25	45	31	15	41	22	34	19
% L_1 / L_2	11	2	7	29	46	81	88	89	96	100	96	98
% L_3	89	98	93	71	54	19	12	11	4	0	4	2
BZ/LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	115	101	101	99	82	95	87	78	111	94	81	84
L_1 / L_2	10	11	9	7	11	30	59	45	77	48	52	53
L_3 Dead	2	4	2	4	2	3	1	3	0	0	0	0
L_3 Alive	65	61	62	50	39	19	6	5	0	0	0	0
Total Larvae	77	76	73	61	52	52	66	53	77	48	52	53
Final egg	38	25	28	38	30	43	21	25	34	46	29	31
% L_1 / L_2	13	14	12	11	21	58	89	85	100	100	100	100
% L_3	87	86	88	89	79	42	11	15	0	0	0	0
AVM AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	137	122	99	77	98	106	109	74	133	101	125	105
L_1 / L_2	6	16	10	10	13	19	57	42	68	58	67	60
L_3 Dead	3	1	1	0	1	1	2	0	0	1	0	0
L_3 Alive	76	69	63	43	47	36	8	9	2	1	0	0
Total Larvae	85	86	74	53	61	56	67	51	70	60	67	60
Final egg	52	36	25	24	37	50	42	23	63	41	58	45
% L_1 / L_2	7	19	14	19	21	34	85	82	97	97	100	100
% L_3	93	81	86	81	79	66	15	18	3	3	0	0

Table B.11
DrenchRite® assay counts for Hc-H992. ** Final egg count included in % L_1/L_2.

Hc-H992	**Wells**											
BZ - A	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	58	55	49	66	69	65	80	75	77	57	75	79
L_1 / L_2	6	3	2	8	10	8	12	11	16	26	44	10
L_3 Dead	1	1	2	0	2	1	2	3	3	4	1	1
L_3 Alive	36	29	39	37	42	34	38	41	25	7	8	2
Total Larvae	43	33	43	45	54	43	52	55	44	37	53	13
Final egg	15	22	6	21	15	22	28	20	33	20	22	66
% L_1 / L_2	14	9	5	18	36	46	50	41	64	81	88	96
% L_3	86	91	95	82	64	54	50	59	36	19	12	4
BZ - B	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	59	52	65	58	83	67	85	83	76	75	58	59
L_1 / L_2	1	3	4	8	8	6	13	20	21	19	26	21
L_3 Dead	0	1	3	1	2	4	2	2	4	6	1	2
L_3 Alive	37	32	43	35	64	44	47	36	32	24	4	0
Total Larvae	38	36	50	44	74	54	62	58	57	49	31	23
Final egg	21	16	15	14	9	13	23	25	19	26	27	36
% L_1 / L_2	3	8	8	18	20	28	42	54	53	60	91	97
% L_3	97	92	92	82	80	72	58	46	47	40	9	3
LEV - C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	58	73	49	43	42	51	62	57	61	48	57	46
L_1 / L_2	2	8	2	7	20	29	36	39	44	39	40	41
L_3 Dead	1	0	1	1	1	0	1	0	0	1	0	0
L_3 Alive	37	53	38	26	10	4	1	3	0	0	1	1
Total Larvae	40	61	41	34	31	33	38	42	44	40	41	42
Final egg	18	12	8	9	11	18	24	15	17	8	16	4
% L_1 / L_2	5	13	5	21	65	88	95	93	100	98	98	98
% L_3	95	87	95	79	35	12	5	7	0	2	2	2
LEV - D	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	56	49	42	43	50	47	36	53	55	46	50	47
L_1 / L_2	2	6	8	5	12	23	23	34	38	36	38	42
L_3 Dead	1	1	1	0	2	0	0	0	0	0	0	0
L_3 Alive	39	31	31	27	9	7	5	4	1	1	0	0
Total Larvae	42	38	40	32	23	30	28	38	39	37	38	42
Final egg	14	11	2	11	27	17	8	15	16	9	12	5
% L_1 / L_2	5	16	20	16	52	77	82	89	97	97	100	100
% L_3	95	84	80	84	48	23	18	11	3	3	0	0

Table B.11 Continued

Hc-H992	Wells											
BZ/LEV - E	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	50	61	55	52	67	55	53	64	66	59	60	44
L_1 / L_2	4	4	4	5	16	27	39	49	49	39	45	30
L_3 Dead	0	0	1	1	2	2	0	0	0	0	0	0
L_3 Alive	35	45	35	38	33	6	1	0	0	0	0	0
Total Larvae	39	49	40	44	51	35	40	49	49	39	45	30
Final egg	11	12	15	8	16	20	13	15	17	20	15	14
% L_1 / L_2	10	8	10	11	31	77	98	100	100	100	100	100
% L_3	90	92	90	89	69	23	2	0	0	0	0	0
BZ/LEV - F	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	48	60	61	70	66	39	54	44	49	56	58	60
L_1 / L_2	9	3	4	6	14	25	39	35	38	37	46	45
L_3 Dead	1	0	0	2	3	0	0	0	0	0	0	0
L_3 Alive	30	28	43	50	31	7	0	1	0	0	0	0
Total Larvae	40	31	47	58	48	32	39	36	38	37	46	45
Final egg	8	29	14	12	18	7	15	8	11	19	12	15
% L_1 / L_2	23	10	9	10	29	78	100	97	100	100	100	100
% L_3	77	90	91	90	71	22	0	3	0	0	0	0
AVM - G	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	61	58	54	52	77	55	62	51	49	57	70	42
L_1 / L_2	7	1	4	3	15	10	33	31	32	45	55	28
L_3 Dead	1	1	1	0	0	0	0	0	0	0	0	0
L_3 Alive	40	45	38	33	44	27	11	5	5	0	0	0
Total Larvae	48	47	43	36	59	37	44	36	37	45	55	28
Final egg	13	11	11	16	18	18	18	15	12	12	15	14
% L_1 / L_2	15	2	9	8	25	27	75	86	86	100	100	100
% L_3	85	98	91	92	75	73	25	14	14	0	0	0
AVM - H	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	47	50	67	50	60	68	67	63	55	80	60	40
L_1 / L_2	2	4	8	3	14	15	33	48	38	62	47	29
L_3 Dead	1	1	1	1	0	1	0	0	0	0	0	0
L_3 Alive	32	36	43	38	41	37	10	3	1	1	0	0
Total Larvae	35	41	52	42	55	53	43	51	39	63	47	29
Final egg	12	9	15	8	5	15	24	12	16	17	13	11
% L_1 / L_2	6	10	15	7	25	28	77	94	97	98	100	100
% L_3	94	90	85	93	75	72	23	6	3	2	0	0

Table B.12
DrenchRite® assay averages for Hc-H992. ** Final egg count included in % L_1/L_2.

Hc-H992	Wells											
BZ AVG	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	117	107	114	124	152	132	165	158	153	132	133	138
L_1 / L_2	7	6	6	16	18	14	25	31	37	45	70	31
L_3 Dead	1	2	5	1	4	5	4	5	7	10	2	3
L_3 Alive	73	61	82	72	106	78	85	77	57	31	12	2
Total Larvae	81	69	93	89	128	97	114	113	101	86	84	36
Final egg	36	38	21	35	24	35	51	45	52	46	49	102
% L_1 / L_2	9	9	6	18	28	37	46	48	58	69	89	96
% L_3	91	91	94	82	72	63	54	52	42	31	11	4
LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	114	122	91	86	92	98	98	110	116	94	107	93
L_1 / L_2	4	14	10	12	32	52	59	73	82	75	78	83
L_3 Dead	2	1	2	1	3	0	1	0	0	1	0	0
L_3 Alive	76	84	69	53	19	11	6	7	1	1	1	1
Total Larvae	82	99	81	66	54	63	66	80	83	77	79	84
Final egg	32	23	10	20	38	35	32	30	33	17	28	9
% L_1 / L_2	5	14	12	18	59	83	89	91	99	97	99	99
% L_3	95	86	88	82	41	17	11	9	1	3	1	1
BZ/LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	98	121	116	122	133	94	107	108	115	115	118	104
L_1 / L_2	13	7	8	11	30	52	78	84	87	76	91	75
L_3 Dead	1	0	1	3	5	2	0	0	0	0	0	0
L_3 Alive	65	73	78	88	64	13	1	1	0	0	0	0
Total Larvae	79	80	87	102	99	67	79	85	87	76	91	75
Final egg	19	41	29	20	34	27	28	23	28	39	27	29
% L_1 / L_2	16	9	9	11	30	78	99	99	100	100	100	100
% L_3	84	91	91	89	70	22	1	1	0	0	0	0
AVM AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	108	108	121	102	137	123	129	114	104	137	130	82
L_1 / L_2	9	5	12	6	29	25	66	79	70	107	102	57
L_3 Dead	2	2	2	1	0	1	0	0	0	0	0	0
L_3 Alive	72	81	81	71	85	64	21	8	6	1	0	0
Total Larvae	83	88	95	78	114	90	87	87	76	108	102	57
Final egg	25	20	26	24	23	33	42	27	28	29	28	25
% L_1 / L_2	11	6	13	8	25	28	76	91	92	99	100	100
% L_3	89	94	87	92	75	72	24	9	8	1	0	0

Table B.13
DrenchRite® assay counts for Hc-GRF. ** Final egg count included in % L_1/L_2.

Hc-GRF					**Wells**							
BZ - A	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	36	46	26	59	46	73	47	41	58	54	44	37
L_1 / L_2	6	4	3	8	5	6	5	7	11	12	11	9
L_3 Dead	1	0	0	0	0	0	1	0	0	2	3	0
L_3 Alive	16	27	15	33	25	41	28	24	30	24	19	4
Total Larvae	23	31	18	41	30	47	34	31	41	38	33	13
Final egg	13	15	8	18	16	26	13	10	17	16	11	24
% L_1 / L_2	26	13	17	20	46	44	38	41	48	52	50	89
% L_3	74	87	83	80	54	56	62	59	52	48	50	11
BZ - B	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	52	69	61	47	47	72	48	42	56	59	56	54
L_1 / L_2	7	5	7	7	9	9	6	5	10	16	7	16
L_3 Dead	0	2	1	1	0	0	0	0	0	3	4	0
L_3 Alive	25	39	35	33	23	39	32	18	33	30	17	5
Total Larvae	32	46	43	41	32	48	38	23	43	49	28	21
Final egg	20	23	18	6	15	24	10	19	13	10	28	33
% L_1 / L_2	22	11	16	17	51	46	33	57	41	44	63	91
% L_3	78	89	84	83	49	54	67	43	59	56	37	9
LEV - C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	55	78	65	46	45	66	57	50	58	74	52	52
L_1 / L_2	8	11	13	8	8	17	12	15	24	28	21	26
L_3 Dead	0	1	0	0	1	2	2	1	2	2	2	4
L_3 Alive	24	42	31	31	26	23	18	19	12	27	14	12
Total Larvae	32	54	44	39	35	42	32	35	38	57	37	42
Final egg	23	24	21	7	10	24	25	15	20	17	15	10
% L_1 / L_2	25	20	30	21	23	40	38	43	63	49	57	62
% L_3	75	80	70	79	77	60	63	57	37	51	43	38
LEV - D	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	69	50	45	42	51	71	75	51	73	46	61	45
L_1 / L_2	10	6	8	7	11	15	16	12	29	13	25	23
L_3 Dead	0	0	0	2	3	1	0	0	2	0	2	3
L_3 Alive	44	33	28	22	25	33	37	20	21	17	11	8
Total Larvae	54	39	36	31	39	49	53	32	52	30	38	34
Final egg	15	11	9	11	12	22	22	19	21	16	23	11
% L_1 / L_2	19	15	22	23	28	31	30	37	56	43	66	68
% L_3	81	85	78	77	72	69	70	63	44	57	34	32

Table B.13 Continued

Hc-GRF	Wells											
BZ/LEV – E	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	101	66	58	77	34	65	59	46	53	65	51	61
L_1 / L_2	12	10	13	11	9	12	7	7	20	42	32	36
L_3 Dead	0	2	1	2	1	2	4	3	3	0	1	2
L_3 Alive	61	39	30	49	20	24	24	9	1	0	3	2
Total Larvae	73	51	44	62	30	38	35	19	24	42	36	40
Final egg	28	15	14	15	4	27	24	27	29	23	15	21
% L_1 / L_2	16	20	30	18	30	32	20	37	83	100	89	90
% L_3	84	80	70	82	70	68	80	63	17	0	11	10
BZ/LEV - F	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	54	66	46	45	55	59	76	54	71	48	47	64
L_1 / L_2	14	9	9	6	8	6	12	16	19	30	32	39
L_3 Dead	0	0	0	0	0	2	3	5	2	0	2	1
L_3 Alive	32	34	23	25	25	31	30	15	10	0	2	5
Total Larvae	46	43	32	31	33	39	45	36	31	30	36	45
Final egg	8	23	14	14	22	20	31	18	40	18	11	19
% L_1 / L_2	30	21	28	19	24	15	27	44	61	100	89	87
% L_3	70	79	72	81	76	85	73	56	39	0	11	13
AVM - G	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	67	53	32	53	44	45	53	55	47	45	68	59
L_1 / L_2	7	10	5	15	11	13	34	41	36	33	38	37
L_3 Dead	1	2	0	1	1	0	2	1	0	0	0	0
L_3 Alive	31	25	20	22	16	18	5	1	0	0	0	0
Total Larvae	39	37	25	38	28	31	41	43	36	33	38	37
Final egg	28	16	7	15	16	14	12	12	11	12	30	22
% L_1 / L_2	18	27	20	39	39	42	83	95	100	100	100	100
% L_3	82	73	80	61	61	58	17	5	0	0	0	0
AVM - H	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	45	46	62	50	51	51	64	40	69	88	64	58
L_1 / L_2	7	10	9	5	14	13	30	29	39	56	41	38
L_3 Dead	0	1	0	0	1	1	2	0	0	0	0	0
L_3 Alive	23	22	38	23	29	20	11	0	2	0	0	0
Total Larvae	30	33	47	28	44	34	43	29	41	56	41	38
Final egg	15	13	15	22	7	17	21	11	28	32	23	20
% L_1 / L_2	23	30	19	18	32	38	70	100	95	100	100	100
% L_3	77	70	81	82	68	62	30	0	5	0	0	0

Table B.14
DrenchRite® assay averages for Hc-GRF. ** Final egg count included in % L_1/L_2.

Hc-GRF	Wells											
BZ-AVG	**1**	**2**	**3**	**4**	**5****	**6****	**7****	**8****	**9****	**10****	**11****	**12****
Starting Egg	88	115	87	106	93	145	95	83	114	113	100	91
L_1 / L_2	13	9	10	15	14	15	11	12	21	28	18	25
L_3 Dead	1	2	1	1	0	0	1	0	0	5	7	0
L_3 Alive	41	66	50	66	48	80	60	42	63	54	36	9
Total Larvae	55	77	61	82	62	95	72	54	84	87	61	34
Final egg	33	38	26	24	31	50	23	29	30	26	39	57
% L_1 / L_2	24	12	16	18	48	45	36	49	45	48	57	90
% L_3	76	88	84	82	52	55	64	51	55	52	43	10
LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	124	128	110	88	96	137	132	101	131	120	113	97
L_1 / L_2	18	17	21	15	19	32	28	27	53	41	46	49
L_3 Dead	0	1	0	2	4	3	2	1	4	2	4	7
L_3 Alive	68	75	59	53	51	56	55	39	33	44	25	20
Total Larvae	86	93	80	70	74	91	85	67	90	87	75	76
Final egg	38	35	30	18	22	46	47	34	41	33	38	21
% L_1 / L_2	21	18	26	21	26	35	33	40	59	47	61	64
% L_3	79	82	74	79	74	65	67	60	41	53	39	36
BZ/LEV AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	155	132	104	122	89	124	135	100	124	113	98	125
L_1 / L_2	26	19	22	17	17	18	19	23	39	72	64	75
L_3 Dead	0	2	1	2	1	4	7	8	5	0	3	3
L_3 Alive	93	73	53	74	45	55	54	24	11	0	5	7
Total Larvae	119	94	76	93	63	77	80	55	55	72	72	85
Final egg	36	38	28	29	26	47	55	45	69	41	26	40
% L_1 / L_2	22	20	29	18	27	23	24	42	71	100	89	88
% L_3	78	80	71	82	73	77	76	58	29	0	11	12
AVM AVG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Starting Egg	112	99	94	103	95	96	117	95	116	133	132	117
L_1 / L_2	14	20	14	20	25	26	64	70	75	89	79	75
L_3 Dead	1	3	0	1	2	1	4	1	0	0	0	0
L_3 Alive	54	47	58	45	45	38	16	1	2	0	0	0
Total Larvae	69	70	72	66	72	65	84	72	77	89	79	75
Final egg	43	29	22	37	23	31	33	23	39	44	53	42
% L_1 / L_2	20	29	19	30	35	40	76	97	97	100	100	100
% L_3	80	71	81	70	65	60	24	3	3	0	0	0

APPENDIX C

Table C.1
The cDNA concentrations (original and dilutions) from NanoDrop Spectrophotometer. The dilution factor is the dilution that was made on the sample to obtain 150 ng per reaction. The volume per reaction is the adjusted volume to obtain 150 ng per reaction.

Sample ID	**cDNA concentration (ng/ul)**	**÷ 150**	**Dilution Factor (1 : X)**	**Dilution concentration (ng/ul)**	**Volume per reaction (µl)**
Hc-OS					
Control 1	652.15	4.35	4.5	131.25	1.14
Control 2	461.53	3.08	3.0	154.46	0.97
Control 3	528.43	3.52	3.5	144.05	1.04
BZ HS 1	570.70	3.80	4.0	107.45	1.40
BZ HS 2	469.15	3.13	3.0	152.61	0.98
LEV HS 1	537.08	3.58	3.5	126.97	1.18
LEV HS 2	543.63	3.62	3.5	128.22	1.17
LEV MS 1	544.45	3.63	3.5	142.17	1.06
LEV MS 2	587.38	3.92	4.0	127.96	1.17
AVM HS 1	573.86	3.83	4.0	112.68	1.33
AVM HS 2	560.14	3.73	4.0	126.98	1.18
Hc-OG					
Control 1	755.74	5.04	5.0	167.03	0.90
Control 2	694.90	4.63	5.0	126.74	1.18
Control 3	576.75	3.85	4.0	135.78	1.10
BZ HS 1	483.13	3.22	3.0	155.76	0.96
BZ HS 2	493.02	3.29	3.5	132.93	1.13
BZ WR 1	552.84	3.69	3.5	137.70	1.09
LEV HS 1	593.26	3.96	4.0	121.54	1.23
LEV HS 2	538.20	3.59	3.5	133.09	1.13
LEV MS 1	515.94	3.44	3.5	134.90	1.11
LEV MS 2	478.72	3.19	3.0	166.28	0.90
AVM HS 1	568.85	3.79	4.0	110.99	1.35
AVM HS 2	540.63	3.60	3.5	113.90	1.32

Table C.1 Continued

Sample ID	cDNA concentration (ng/ul)	÷ 150	Dilution Factor (1 : X)	Dilution concentration (ng/ul)	Volume per reaction (µl)
Hc-EG					
Control 1	556.36	3.71	4.0	129.37	1.16
Control 2	637.51	4.25	4.5	123.27	1.22
Control 3	541.05	3.61	3.5	140.54	1.07
BZ HS 1	506.29	3.38	3.5	128.84	1.16
BZ WR 1	477.91	3.19	3.0	154.82	0.97
LEV MS 1	524.48	3.50	3.5	140.11	1.07
AVM HS 1	535.91	3.57	3.5	139.96	1.07
AVM WR 1	523.81	3.49	3.5	142.81	1.05
Hc-RFR					
Control 1	486.22	3.24	3.5	137.08	1.09
Control 2	697.67	4.65	4.5	134.14	1.12
Control 3	527.50	3.52	3.5	129.91	1.15
BZ HS 1	518.68	3.46	3.5	137.05	1.09
BZ WR 1	425.40	2.84	3.0	141.91	1.06
BZ WR 2	476.19	3.17	3.0	159.87	0.94
BZ HR 1	564.28	3.76	4.0	120.39	1.25
LEV MS 1	481.46	3.21	3.5	137.52	1.09
LEV MS 2	549.14	3.66	3.5	148.41	1.01
AVM HS 1	461.65	3.08	3.0	150.58	1.00
AVM WR 1	506.89	3.38	3.5	144.98	1.03
Hc-H992					
Control 1	613.91	4.09	4.0	132.79	1.13
Control 2	607.36	4.05	4.0	130.38	1.15
Control 3	488.74	3.26	3.5	125.74	1.19
BZ HS 1	616.21	4.11	4.0	128.74	1.17
BZ WR 1	483.58	3.22	3.5	137.64	1.09
BZ WR 2	548.08	3.65	3.5	144.34	1.04
BZ WR 3	524.58	3.50	3.5	126.55	1.19

Table C-1 Continued

Sample ID	cDNA concentration (ng/ul)	÷ 150	Dilution Factor (1 : X)	Dilution concentration (ng/ul)	Volume per reaction (µl)
Hc-H992					
BZ WR 4	480.52	3.20	3.5	129.30	1.16
BZ HR 1	504.86	3.37	3.5	136.36	1.10
LEV HS 1	547.53	3.65	3.5	128.12	1.17
LEV MS 1	572.96	3.82	4.0	123.97	1.21
LEV MS 2	574.43	3.83	4.0	127.22	1.18
AVM HS 1	519.82	3.47	3.5	136.49	1.10
AVM WR 1	500.04	3.33	3.5	142.26	1.05
Hc-GRF					
Con 1	426.03	2.84	3.0	151.92	0.99
Con 2	468.40	3.12	3.0	164.98	0.91
Con 3	431.70	2.88	3.0	151.21	0.99
BZ SS	448.72	2.99	3.0	159.84	0.94
BZ HR	445.48	2.97	3.0	154.60	0.97
LEV SS	451.38	3.01	3.0	155.95	0.96
LEV HR	445.17	2.97	3.0	161.92	0.93
AVM HS	402.68	2.68	2.5	170.09	0.88
AVM WR	412.15	2.75	3.0	142.14	1.06
Positive control	143.19	0.95	N/A	N/A	1.05

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