Potential Transmission of Pathogens Between Human Hosts by the Hookworm, *Necator Americanus*

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ABSTRACT

Experimental trials are underway exploring the use of parasitic helminths to treat autoimmune diseases. As with any experimental therapy, such trials must adequately address safety concerns associated with the treatment protocol. In the treatment protocol employed by Autoimmune Therapies, patients are inoculated intradermally with the common nematode hookworm *Necator Americanus*. Beyond the potentially pathogenic effects of hookworm infection itself, there are also concerns as to whether the hookworms might act as a vector for secondary infectious pathogens such as viruses, bacteria or fungi. This is of particular concern given that hookworm larvae are cultured from the feces of previously inoculated individuals, under non-sterile conditions. In order to address the possibility of treatment-associated transmission of pathogens by *N.Americanus,* an extensive review of the current knowledge is presented. In this survey, no evidence implying potential transmission of human viral pathogens by *N.Americanus* was uncovered. Additionally, no evidence for the transmission of bacterial pathogens by hookworms was uncovered. There are, however, a number of examples of bacterial transmission between human hosts by the filial nematodes. Additionally, some *in vitro* studies have demonstrated infection of the nematode *C.elegans* with human pathogenic bacteria, and there is some evidence for transmission potential. Thus, although there are no known pathogenic bacteria that infect *N.Americanus*, the transmission of such pathogens between hosts cannot be ruled out. Finally, though there are no studies examining fungal associations with the hookworm, some fungi with established human pathogenesis are infective in other nematodes *in vitro*. In summary, though there is very

little evidence to suggest possible transmission of pathogens by hookworms, future studies are required to rule this out unequivocally.

I. VIRAL TRANSMISSION BY NEMATODES

Known Viruliferous Nematodes

Nematodes act as the principle vector for the plant viruses *Nepovirus* and *Tobravirus*. These are noncirculative viruses that do not travel across cell membranes and are unable to replicate within the vector. Noncirculative viruses are carried only externally on the cuticle lining of the vector's mouthparts or foregut. During transmission, viral particles dissociate from the vector and are directly injected into the host plant root cells via the nematode feeding structure. Noncirculative transmission is not common among vector borne animal viruses. Among the nematode-borne plant viruses, retention and dissociation regulates the specificity of the interaction, and different nepoviruses are only transmitted by certain species of nematodes. Importantly, since noncirculative viruses are carried extracellularly on the vector's cuticle, the virus is shed along with the cuticle during each molt. The virus-vector interaction as described in nematodes is therefore nonpersistent or semipersistent, in that it is nonreplicative and transient (1).

Water-borne Nematodes as Passive Viral Vectors

In a public health study conducted in 1960, investigators examined the ability of rhabditic nematodes to serve as carriers of human pathogens (2). A previous finding of nematodes in a city water supply had raised concerns that the worms could be internalizing pathogens and shielding them from chlorine during water purification. Researchers cultured worms in the presence of the enteric viruses Coxsackie A9 and Echo7 (both single-stranded RNA viruses), then washed away free virus and prepared

worm extracts. Treated cells were incubated with the extracts in order to assay the cytopathic units of virus that had been internalized by feeding worms. After 2 hours, there was 100% survival of pathogenic virus; this was reduced to 15% after 24 hours and <1.67% after 48 hours. This suggests that virus internalized by the worms remained viable up to 48 hours. To determine whether viable virus could be excreted, live worms were co-cultured with cells for 48 hours. No cytopathic changes were observed in any of the treated cells, indicating that no viable virus was excreted by the nematodes. The authors conclude that though rhabditic nematodes could ingest and internalize viable virus, there was little potential for them to serve as a pathogen carrier. Given the rapid loss of pathogen viability within 48 hours of ingestion, the authors further propose that water-borne nematodes could only potentially transfer virus to a water supply if the transmission occurred within 24 hours of ingestion (2). [1]

Viral Infection of *C.elegans In Vitro*

No viruses have been reported to infect *C.elegans* or *N.Americanus*. The roundworm *C.elegans* is a non-parasitic nematode that is a highly studied model organism and closely related to *N.Americanus* (3). It is a simple target for genetic manipulation, its genome has been completed and well-annotated, and it possesses a relatively small number of cells that are easily visualized in through its transparent cuticle, making it ideal for studying cellular processes at the molecular level. Whereas this organism is frequently employed in studies of bacterium-host interactions, the

[1] The authors also evaluated the transmission of pathogenic bacteria by these nematodes, with similar results. Importantly, since the publication of this article in 1960 it has been cited only five times, all of which refer only to the bacterial findings. This strongly implies that no contradictory findings, establishing nematodes as a vector for human pathogens, have emerged since this time.

absence of a known viral agent capable of infecting *C.elegans* has rendered it unsuitable for virological studies. In a recent study, researchers attempted to infect *C.elegans* in vitro with vaccinia virus in order to establish the use of this model organism for studying virus-host interactions (4). A wild-type strain of *C. elegans* could not be infected with vaccinia virus (VV), Sindbis virus, baculovirus, or adenovirus by a number of standard infection protocols. The authors sought to determine whether the insusceptibility of *C. elegans* to VV infection was due to the lack of extracellular virus receptors or intracellular impediment for viral replication in *C. elegans*. PEG is a hydrophilic molecular crowding agent that increases viral uptake efficiencies by increasing membrane permeability(5, 6, 7). The addition of polyethylene glycol (PEG) to the infection protocol permitted transmission of VV to L3 and L4 worms at an infection rate of 5%. Following PEG treatment, the infected worms were shown to contain actively replicating virus as detected by a lacZ reporter assay, and replication was detected up to three days post-infection. Additionally, viral particles were detected by EM, also implying that active viral replication and assembly is possible following infection. Sindbis virus was also shown to be infective and replication-competent in 10% of PEG-treated worms. The dependence of VV infectivity on PEG pretreatment implies that though viral entry is prevented in untreated cells, the cells remain permissive to viral replication. The authors also examined both eggs and progeny larvae from VV-infected adults and found no evidence of viral infection, indicating that vertical transmission was not occurring (4).

Antiviral Defense Mechanisms in *C.elegans*

Interestingly, following PEG-mediated infection of *C.elegans* with VV, viral replication efficiency was found to increase markedly in animals carrying mutations in *ced-3*, *ced-4*, *ced-9* or *egl-1* genes (4). These genes are core components of PCD, the programmed cell death response pathway. Programmed cell death, or apoptosis, is an important process in normal development and is regulated by a cascade of proteases that degrade essential cellular proteins, resulting in necrotic cell death. The genes encoding these proteases are highly conserved among eukaryotes. Misregulation of PCD in mammals is associated with a number of human diseases, including cancer, neurodegenerative disease, AIDS and other autoimmune diseases (8). This study implies that the core PCD genes in *C.elegans* play a role in restricting the replication of VV.

RNA interference (RNAi) is an evolutionarily conserved mechanism for silencing gene expression, and is believed to function as an innate antiviral immune response. Following viral infection, RNAi is triggered by the presence of double-stranded RNAs, and this results in the downstream degradation of target viral mRNAs, and post-transcriptional gene silencing (PTGS) A number of genes encoding proteins in the RNAi pathway have been characterized in *C.elegans*, and it has been proposed that this pathway also provides antiviral immunity in the worm. Recently, two groups demonstrated *in vitro* viral replication in cultured primary cells from *C.elegans*. Recombinant vesicular stomatitis virus (VSV), a virus with a broad range of hosts including mammals and insects, was able to infect and replicate in primary *C.elegans* cells in culture, though the infection did not produce infective progeny. In cells isolated from worms carrying mutations in the RNAi pathway, VSV infection rates doubled and increasing viral titers

were observed, indicative of a productive infection (9, 10). The second group used a transgene carrying the Flock-house virus (FHV), another promiscuous virus capable of infecting a wide spectrum of hosts (including humans), to infect *C.elegans* primary cells. The authors showed that FHV infection involved an antagonistic interaction between *rde1*, a cellular component of the RNAi pathway, and B3, a virally encoded inhibitor of RNAi. Whereas infection was inhibited when viral B3 was mutated, infection rates increased significantly in *rde1* mutant worms. Taken together, these studies provide evidence that RNAi and programmed cell death both act as authentic antiviral defense mechanisms in the worm (4, 8,10, 11). These studies establish the use of *C.elegans* as a model for studying virus-host interactions. The infection protocols described in these studies require extensive manipulation of culture conditions *in vitro* and in the absence of any known viral pathogens capable of infecting *C.elegans*, there remains very little known regarding *in vivo* viral infection of *C.elegans*.

In its human host, the adult hookworm *N.Americanus* resides in the small intestine and eggs are passed into the stool, where they hatch after leaving the host's body. After hatching, the larvae molt twice over the next ten days prior to attaining infectivity at larval stage 3 (L3). In order for the hookworm to act as a viral vector, the virus must be transmitted from the human host to a feeding adult worm. Once inside the adult, it must then be capable of vertical transmission to the progeny. In the studies investigating *in vitro* viral infection of *C.elegans*, no vertical transmission was observed (4). The only known *in vivo* virus-vector interactions in nematodes are the semipersistant, noncirculative viruses transmitted by plant-feeding nematodes. Since viral infection is

extracellular and does not persist after molting, even virus capable of vertical transmission in this host would not persist to the infectious larval stage. In the *N.Americanus* culture protocol employed by Autoimmune Therapies, only the eggs are collected from inoculated individuals, and adult worms are not cultured. Following collection, the eggs are incubated for the time required to obtain infective larvae, and have undergone two molting cycles. This prevents the exposure of the patient to any stage of the hookworm lifecycle capable of transmitting pathogenic viruses.

II. NEMATODAL VECTORS OF BACTERIAL PATHOGENS

Water-borne Nematodes as Passive Viral Vectors

In the previously described study of water-borne rhabditic nematodes, Chang et al. studied whether worms could harbor and excrete viable bacterial pathogens (2). The nematodes were able to feed on the human enteric bacteria Salmonella (*S. typhosa, S. paratyphi* and *S. typhimurium*) and Shigella (*Sh. Sonnei* and *Sh. Dysenteriae II*), and 0.1-1% of the bacteria remained viable after 48 hours in the worm gut. However, after worms were plated onto bacterial growth media no colonies were detected after 5 days of culture, demonstrating that no viable bacteria were excreted from living worms. As was determined with ingested virus, the rapid loss of pathogen viability in the worm gut and absence of viable bacteria in worm excreta led researchers to conclude that transmission of enteric bacteria could only potentially occur within 24 hours of ingestion. This indicates that in these worms, vertical transmission of the enteric bacteria *Salmonella* and *Shigella* from adult to progeny would not be possible (2).

Symbiotic Associations Between Nematodes and Bacteria

In a well-characterized example of biological symbiosis, the nematode *Steinernema* can harbor and transmit the pathogenic bacteria *Xenorhabdus* between insect hosts. This mutualism is a highly specialized interaction between specific strains of *Steinernema* and *Xenorhabdus*, and no bacterial strain was capable of associating with a species of *Steinernema* phylogenetically distant from its natural symbiont (12, 13, 14). This also implies that these organisms have co-evolved and that the association is evolutionarily beneficial. *Steinernema* infective juveniles live in the soil and infect

insects, such as certain moths and beetles, colonizing the body cavity. Once inside the cavity, the juveniles mature, shed their cuticle, penetrate the midgut wall and release infectious *Xenorhabdus* bacteria into the insect hemocoel (15). The insect dies rapidly, and the nematodes reproduce within the cadaver. Newly produced infective juveniles then feed on the insect remains and thus reassociate with the bacteria, before leaving the cadaver and entering the soil (16). This is considered pseudo-vertical transmission, since the transmission of bacteria between adult and offspring is indirect (17). In the juvenile worm, the bacteria are transported and replicate in a special organ within the intestine known as "the vesicle of Bird and Akhurst" (16, 18). Following reinfection of a new insect host, the bacteria remain inside this intestinal vesicle until the juvenile worm enters the digestive tract of the host, at which point the bacteria migrate towards the anus and, after maturation is complete, are ultimately released (13). An analogous symbiotic lifecycle is seen in a closely related entomopathogenic (insect-killing) nematode, *Heterorhabditis bacteriophora* which serves as a vector for the bacteria *Photorhabdus luminescens* (19).

The filarial nematodes have symbiotic relationships with the bacterial genus *Wolbachia* (20). These nematodes, including *Onchocerca volvulus*, *Wuchereria bancofti* and *Brugia,* are pathogenic to mammals, and in humans they are responsible for river blindness, elephantitus, and lymphoedema, respectively. *Onchocerca volvulus* is carried by the blackfly, whereas *Wuchereria bancofti* and *Brugia* are carried by mosquitos. In each case, the L3 larvae enter the host bloodstream through the insect bite, and then migrate subcutaneously within the lymphatic system where they will mature into adults.

After mating, the female sheds L1 microfilariae which are then transmitted to the insect host during feeding. Inside the host, the larvae will shed twice more before reaching L3 infectivity. It is believed that the pathogenicity of infection is due to the host immune response to the bacteria, leading to large-scale inflammation and tissue damage (21). *Wolbachia* has been shown to localize within oocytes, embryos, and L1 microfilariae within the reproductive tract of adult female nematodes, suggesting that the bacteria can be transmitted vertically from adult to progeny (22). Interestingly, treatment of patients infected with either *Onchocerca* and *Wuchereria* with the antibacterial antibiotic doxycycline reduced the levels of *Wolbachia* bacterial infection but also left the adult female worms sterile, suggesting that the symbiosis between worm and bacteria is obligatory. The authors describe a treatment protocol using the anti-helminth Ivermectin in combination with doxycycline in order to rid patients of microfilia-associated disease (23,24).

Bacterial Infection of *C.elegans In Vitro*

Though a natural viral infection of *C.elegans* is unknown, this organism is a well-established model for bacterial pathogenesis. Many human bacterial pathogens can infect *C.elegans.* Bacteria with established pathogenesis in both humans and nematodes include *Salmonella enterica*, *Pseudomonas aeruginosa*, *Escherichia coli, Serratia marcescens, Streptococcus pneumoniae*, *Staphylococcus*, *Enterococcus faecalis,* and *Shigella* (25-27). These agents are transmitted to *C.elegans* during feeding in culture *in vitro*. Some of the bacterial pathogens kill through a toxin-mediated mechanism (e.g. *Pseudomonas*) whereas others establish a persistent infection in the gut and exploit

cellular signaling pathways to induce lethal changes in the host's cellular structure (e.g. *Shigella, Salmonella*) (25,28-30). When fed to worms *in vitro*, enteropathogenic *Escherichia coli* is able to colonize the posterior intestine of *C.elegans*, and kills the worm within approximately 7 days of exposure (see (27) for review). *Shigella* and *Salmonella* strains have been shown to accumulate in the intestine of the worm during feeding and, following infection, almost 100% of the worms are killed within 10 days (26). It should also be noted that *C. elegans* has been demonstrated to transport *Salmonella* from the soil to fruits and vegetables, though this transmission is thought to occur passively, causing only surface contamination of crops (31-33). Co-culturing worms previously fed infectious *Salmonella* with uninfected worms resulted in transfer of the pathogen to naïve worms. Importantly, *Salmonella* was also isolated from worm progeny two generations subsequent to infection (33). This study demonstrates that bacterial pathogens can be transmitted both horizontally between feeding adults, and vertically from adult to progeny.

Because of the hookworm lifecycle, in order for a bacterial pathogen to be transmitted between human hosts by a hookworm vector, the pathogen must be transmitted vertically. Pseudo-vertical transmission of bacteria in nematodes requires the transmitting adult to shed into the hemocoel, where the feeding juveniles will ingest the pathogen. In *N.Americanus*, the adult and infective larval stages never reside nor feed within the same tissue systems, thus making pseudo-vertical transmission of bacteria impossible. Transmission of *Wolbachia* by the filarial nematodes and was demonstrated to occur vertically. The presence of bacteria in the reproductive cells was required for the

worm's fertility, providing further evidence for the symbiotic coevolution of vector and bacteria (22). Though no symbiotic bacterial association with *N.Americanus* has been demonstrated, this cannot rule out that there are native bacteria utilizing the hookworm vector that have yet to be identified. Vertical transmission of *Salmonella* was also demonstrated in *C.elegans*, and though bacterial infection was not demonstrated in the eggs, presumably eggs harboring the bacteria develop into infected larvae (33). This suggests it would be possible for bacteria ingested by feeding hookworms to be transmitted between hosts, even though the infectious larval stage is entirely physically isolated from the adult worm. Given this, although no human pathogenic bacteria have been identified in association with *N.Americanus,* a study of the potential of hookworm to act as a vector for *Salmonella* and other human enteric bacteria is certainly warranted.

III. FUNGAL TRANSMISSION BY NEMATODES

There are a number of types of interactions between nematodes and fungi, including nematodes that feed on fungi, fungi that predate or parasitize nematodes, and nematodes behaving as passive vectors for the transmission of fungal spores. Though a number of these interactions are well described in plant-associated nematodes, there is very little known about the potential for nematodes to spread pathogens among animal hosts. The fungal genus *Malassezia* is a potential human pathogen that can cause skin disease, and has been found in association with soil nematodes. A correlation between nematodal infestion in the soil and the appearance of *Malassezia*-related otitis in cattle has been established, providing anectodal evidence for the worm's ability to act as a vector for transmission of pathogenic fungi (34). A recent study screened three species of European soil nematodes for the presence of fungal DNA. The protocol amplified DNA from complete worm extracts, and thus would not distinguish between feeding-associated presence of intestinal fungi and parastic fungal infection within the worm. *Malassezia* fungi were detected within two different species of soil nematodes, *Malenchus* and *Tylolaimophorus typicus*, though not in a third species, *Prionchulus*, and the authors propose this is due to the carnivorous status of the this species. If the absence of association of fungi with *Prionchulus* is diet-dependent, this would suggest *Malassezia* fungi is being ingested by the other nematodes. The reason for the detected association is therefore likely due to nematodal feeding habits, and not fungal predation or a mutualistic relationship between these organisms (35). There is as yet no evidence for vertical transmission of *Malassezia* fungi between hosts.

There are two examples of fungal organisms with pathogenicity in humans that have been shown to establish infection in *C.elegans in vitro*. *Cryptococcus neoformans*, a virulent pathogen among immunocompromised people, can colonize the intestine in *C.elegans* and kill the worm within about 7 days of infection (36, 37). *Candida albicans*, also a serious health concern in immunocompromised patients, colonizes the intestine in *C.elegans* and produces characteristic pseudohyphal projections that kill the worm by puncturing the worm cuticle (38). In both cases, worm mortality was complete, preventing any study of vertical transmission of pathogen. Almost all fungal species with established pathogenicity in humans can cause systemic infection in immunocompromised hosts. In such cases, there would be a potential for fungal spores to be present in the intestine. Spores could then be passively transferred to a feeding adult hookworm via the worm's external cuticle, though in such a scenario vertical transmission would be impossible. It is also possible that the fungal spores could be ingested by the feeding worm, and colonize the intestine. Whether colonization of the worm gut could lead to vertical transmission via infectious larvae is unknown. It should be added that no examples of vertical transmission of fungi between nematodes of any species has been documented, making the transfer of pathogenic fungi between human hosts extremely unlikely.

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