Research Article ISSN 2639-9458

Microbiology & Infectious Diseases

An Overview of Guinea Worm Disease and Analysis of its Potential for Global Eradication by means of Diagnostic Assay Development

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Received: 08 February 2020; **Accepted:** 04 March 2020

Citation: Mary C. Blake MS, CRA. An Overview of Guinea Worm Disease and Analysis of its Potential for Global Eradication by means of Diagnostic Assay Development. Microbiol Infect Dis. 2020; 4(1): 1-6.

Keywords
Tropical diseases, Guinea worm, Parasites.

Introduction
Guinea worm disease is one of the known neglected tropical diseases that is near eradication yet still afflicts humans. Guinea worm is transmitted by the nematode Dracunculiasis medinensis and is transmitted exclusively to humans via drinking water contaminated with infected copepods [1]. After infection, the female worm induces a painful blister on the skin, mostly from the lower limbs; the blister ruptures, and larvae are released on contact by the adult female worm as it emerges [2].

Drinking unfiltered water from stagnant water sources containing the infected Cyclops perpetuates the transmission. The transmission cycle can be interrupted at different points by avoiding contaminated sources of drinking water, filtering unsafe water with cloth and fine-mesh strainers before consuming, drinking water from improved sources and controlling the vectors of transmission. Parasitic worms are multicellular organisms with defined anatomical features such as feeding and reproduction [23]. It appears that different cell types and immunoglobulin isotypes are active against different developmental stages of parasites. Eosinophils are more effective at killing newborn larvae, whereas macrophages are very effective against mature extracellular worms. Antibodies that block particular orifices such as mouth or genitalia interfere with critical physiological functions and may cause starvation or curtail reproduction. Currently, no vaccine is available for prevention or medicine for treatment [1].

An immunological test capable of diagnosing early prepatent infections would be useful, as there is little evidence of any chemotherapeutic agents that are active against these parasites [2]. Additionally, it would be useful to have an inexpensive and effective agent capable of killing larvae inside adult female worms before their emergence to forgo the impending debilitation caused by the disease. Many federal, private, and international agencies are helping the countries that still have endemic Guinea worm cases to eradicate this disease [2,3].

Cases have decreased from 3.5 million per year in 1986 to 22 in 2015. Once eradicated, guinea worm will be the first parasitic human disease to be extinct, and the first eradication campaign to be carried out and successfully concluded without vaccine or medicine, solely by using public – private sector partnerships to fund and support community level interventions and innovative incentives to empower exceptional community involvement [3]. The following didactic will commence of a literature review overviewing the knowledge currently understood about Guinea worm disease and will highlight the reasoning why an early diagnostic assay is imperative to achieve permanent eradication of the affliction without the possibility of re-occurrence [4].

Literature Review
Background and Significance
Life Cycle and Structure- The parasite undergoes a life cycle comprised of 6 developmental stages [1,2]: When larvae are released into open water they are ingested by copepods, which are a vector of disease. The larvae molt twice inside of the gastrointestinal tract of the copepods, and become infective larvae with 2-3 weeks, at which time either they are expelled from copepod (micro-crustacean), or the infected copepod is ingested and dies releasing D. medensis larvae into an open body of water where they are ingested by human hosts [5].

After ingestion, the copepods die and are digested inside of the host, this releases the stage 3 larvae, which then penetrate the host's stomach or intestinal wall, and then enter into the abdominal

cavity of the retroperitoneal space [2]. After maturation which takes approximately three months, copulation occurs and the male worms die after mating and is absorbed then excreted by the host's body. Adult females produce millions of eggs. She migrates down to the lower limbs of the body where she formulates painful blisters, inflammation, and localized redness as she begins to emerge from the host's body. This burning pain makes humans want to soak in water [5-7]. Once they enter a lake or pond the female releases her eggs, thus restarting the cycle. Death of adult female worms inside of the body can lead to arthritis and even paralysis of the spinal cord [2].

Disease Morphology and Management

Immunology

Antibody- dependent cell- mediated cytotoxicity has been shown to play a part in parasitic infections. Helminths are manipulative immune-regulators with characteristic Th2- dominated responses [8]. When effector responses are muted in the hosts' body, the parasite is able to persist and propagate by modifying their external environment, indicating that a state of active suppression is mediated by the parasite. There are some drug treatments that can reverse this process, however for GWD there is no effective treatments thus far. Hence, research on valid GWD detection is needed to focus on excretory- secretory products released by live parasites, which can interfere with any aspect of human immunity from initial recognition to end state emergence [4].

Then using bioinformatics, predictions of secreted proteins on the basis of signal peptide sequences can be located. In most parasitic infections the humoral immune response is the main protective factor against extracellular pathogens. Typically, antibodies bind to accessible antigens on the surface of the microbes which act as an opsonin increasing the likely-hood of phagocytosis and clearance of the microbe [9]. Additionally, the binding of complement can lead to direct lysing of the parasite. Unlike microbial pathogens, the immune responses of hosts to worm infections are similar [8,10]. There is generally high levels of IgE, as well as eosinophil and mast cell responses. This reflects the production of significant quantities of IL-4 which induces B cells to class switch to IgE production and stimulates the growth of mast cells, IL-5 which causes induced bone marrow precursors to differentiate into eosinophils, and IL-13 which is secreted by Th2 cells and plays a role in the auto-inflammatory process against protozoan infections [11].

In addition to specific T dependent responses, non-specific hypergammaglobulinemia is present in many parasitic infections. Much of this non- specific antibody is the result of polyclonal B cell activation by released parasite antigens acting as mitogens. This response is ineffective at counteracting the parasite and can enhance the pathogenicity by causing the production of autoantibodies, and my actually lead to diminished specific response due to B cell exhaustion. Some parasite molecules act as Tcell mitogens. This could lead to the generation of autoreactive T cells or activation of suppressor responses [23]. IL-13 specifically induces physiological changes in parasitized organs that are required to expel the offending organisms or their products. Typically, once most worms enter the gastrointestinal tract, expulsion requires IL-13 secreted by Th2 cells. IL-13 induces several changes in the gut that create an environment hostile to the parasite, including enhanced contractions and glycoprotein hyper-secretion from gut epithelial tissues, that ultimately lead to detachment of the organism from the gut wall and their removal [10,11].

Therefore, the immune responses to these helminth species exemplify distinct functions of protective TH2-type immune responses, one leading to worm expulsion and the other contributing to the control of pathological inflammation [8]. The effector molecule responsible for parasite killing can be enhanced by the presence of lFNy and other TH1 cell- promoting cytokine release.

Challenge

Interestingly, in the case of guinea worms, there is actually a reduced Th2 cytokine response [12]. Guinea worms illicit very little immune reactions from the host while still inside of the body, there is speculation that rather than allocate inflammation, the worms inhibit inflammatory responses by down regulating the cytokine signaling cascade of events which could be counter protective to the host [10]. The body begins to violently respond as the adult female exits the skin a year after infection, yet, little is known about the host immune response process [12].

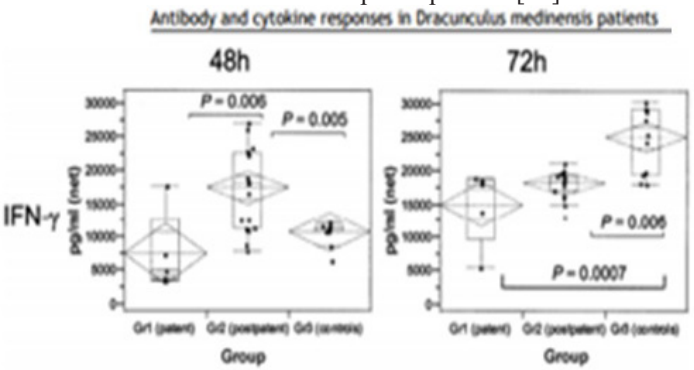

Figure 1: IFNy- Analysis of the cellular cytokine response in Dracunculus patients showed an antigen specific decrease in IFNy production with patent infection. IFNy- are depressed the controls are starting to have more response after 72hr it was higher at 48hr for post patent, from 72hr it almost reverses.

Clinical Presentation

Guinea worm disease is rarely fatal, however it does cause a degree of severe disability from the painful ulcers and abscesses, which occur on the legs and feet which can last for several months, or longer if there is infection with more than one worm [13]. Approximately 0.5% of sufferers become permanently disabled if septic arthritis causes the joints to lock and deform, or ankylosis which is an abnormal stiffening and immobility of joints due to fusion of the bones. Guinea worm presents as a large painful blister tender around the skin were the adult female worm later emerges, however, the disease can present with worms emerging from any part of the body including the back, chest, abdomen, breasts, testicles, pancreas, or spinal cord [2,5,14,15]. There could also be

allergic symptoms manifestation with the discharge of larvae. The emergence of the worm is accompanied with painful edema, intense generalized pruritus, blistering, and ulceration [9]. This pain may incapacitate the affected person for a period of 8 weeks to 2 years. If the worm is broken off before it is completely extruded from the skin, it could cause an extreme inflammatory response with permanent scarring or deforms from debilitating muscle and joint pain, as seen in figure 1 [5]. Other chronic complications include encapsulation of the adult worm may occur when calcified remains of the worm persists in the extremity of the patient.

Challenges

There are other human lesions my mimic guinea worm diseases hence making laboratory confirmation necessary during the early stages of infection, but none exist.

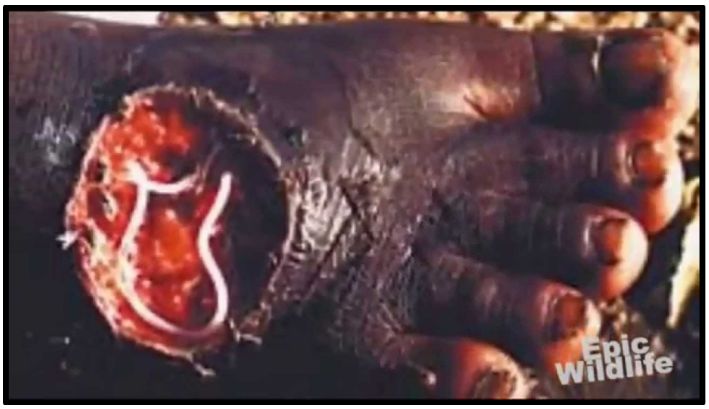

Figure 2: Picture of live Guinea worm inside ulcerated foot (Dracunculus medinensis).

Treatment

Examination of protective immunity for Guinea Worm Disease in human populations has been extraordinarily difficult. Population-based studies have identified groups of individuals who appear to be infection free despite long term exposure to the parasites. Therefore, development of prophylactic strategies and treatments against parasites require a profound knowledge of the responses of immune and non-immune human tissue to infection [23]. Preventative measures from infection include stopping people from drinking water contaminated with copepods by providing safe sources such as wells, filtering water through a fine mesh, boiling, or treating water sources with a larvicide to kill copepods [2]. Furthermore, those with emerging guinea worm are not permitted to enter water sources. Once infected the only therapy is the slow extraction of the emerging female worm by winding it around a stick which can take weeks during which time the patient may be too incapacitated to carry on activities of daily living [1,2,6]. Other chemical controls include Mebendazole or organo-phosphorus compounds that have been widely used to kill infectious larvae in potable water sources and have been shown to have good residual effects and be safe to humans [16,2].

However, this process is expensive and must be repeated monthly. The problem is that there is no immunological test capable of diagnosing early pre-patent infections, as there is evidence that some chemotherapeutic agents are active against developing parasites. There is no drug to treat Guinea worm disease and no vaccine to prevent infection [16]. There have been previous experiments studying the effect of ivermectin on prepatent guinea-worm infections tested in a single-blind placebo-controlled trial. It composed of 400 adults randomly allocated a single dose of ivermectin (150 μg/kg) or placebo [17]. Unfortunately, the drug had no effect on prepatent guinea worms. No one is immune to Guinea worm disease. People in affected villages can suffer year after year [2,7].

Challenges

There is some controversy about the use of organo-phosphates in drinking sources [5]. Although it is claimed to be safe for consumption, there is some draw back for its use because there has been few studies on its effects to the human body, specifically to young children. Also, the use of ivermectin as a chemotherapeutic has been shown to be largely ineffective offering no alternative solutions [17].

Disease Burden and Epidemiology

Socioeconomic effects on communities

Guinea worm affects rural populations whose livelihood depends on farming [18,7]. Since the disease causes disability either temporary or chronic, patients are often prevented from farming. The economic burden of disability among communities of self-employed farmers is especially detrimental due to the inability of these farmers to harvest and sell their crop [15,19]. The effects of not being able to work on villages agricultural output, also acquires considerable loss as households cannot support themselves and rely on the local government for assistance. Many of these local governments struggle to provide adequate sustenance while the family suffers from GWD. Likewise, afflicted children often miss school and lessons which prevent them from valuable knowledge and put them behind in class making the economic costs very high for the community. Finally, there is a negative social stigma of those afflicted with GWD that they or their crops are less than desirable because of it's potential of being infected with parasitic worms. In the 1940's over 48 million people were estimated to be affected by the disease in Africa, India, and the Middle East. During the 1980's an estimated 3.5 million cases were reported annually [3]. As a result of the efforts of the National Guinea worm eradication program funded by the Carter Foundation, 17 countries have eliminated the disease, and transmission remains endemic to only four. Chad, Ethiopia, Mali, and South Sudan. A total of 126 cases of Dracunculiasis were reported in 2014 down more than 99% [7].

Challenge

Insecurity that prevents full program roll out in some parts of Sudan, Mali and Chad with nomadic movement of populations within and outside of national borders [15]. The need for crucial research is underway to investigate this transmission dynamics in order to identify appropriate ways to accelerate the interruption of the transmission cycle. Research is needed to understand the

dynamics of guinea worm infection during the course of its year long incubation, and finally, to address the operational challenge of vector control in large bodies of water.

Control Tools and Eradication Strategies

The development of the Dracunculiasis eradication strategies were based on evidence gathered from various studies on the disease, the effective interventions and lessons learned from the smallpox eradication program [5]. The strategies adopted for the eradication program include, interrupting the transmission of the disease, filtering pond or river water to remover copepods, boiling water, and using larvicide such as Temphos. In addition, there are several strategies allocated to address the methods of eradication proposal, such as surveillance of prompt detection of cases, reporting these cases to healthcare professionals, and provision of general education to the public [2,5]. If the transmission of a new case is suspected, the eradication process goes through the following stages: locating endemic regions, interruption of transmission, and increasing community awareness. That is followed by a precertification stage which lasts three years, during which time the country must maintain consistent surveillance to demonstrate evidence of continued absence of transmission in order to be qualified for certification as Guinea worm disease free. Once the World Health Organization certifies a country free of guinea worm, it enters the post certification phase which is continued until eradiation is declared globally [5].

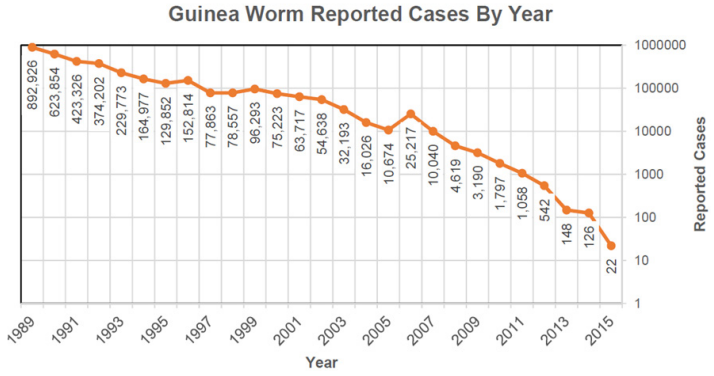

Figure 3: Logarithmic scale of reported human cases of guinea worm by year, 1989–2015 (2015 data is provisional). Data from Guinea Worm Eradication Program.

Eradication programs announce suitable cash rewards for voluntary reporting of cases. In the 1940's more than 48 million people were estimated to be affected by the disease in Africa, India, and the Middle East. By the 1980's the disease was known to be endemic in 20 countries[19] [7]. As seen in figure 3, cases of guinea worm disease has been steadily decreasing over the past five years. The Global Eradication Campaign funded by the Carter Foundation [3], has continued to progress steadily towards wiping guinea worm disease off the face of planet as seen in figure 3. In fact there has been a 98% reduction in GWD reported cases since 1946 [6]. Case management is continued by preventing infected persons from wading into drinking water sources, and by instructing providers that other human lesions might mimic Guinea worm disease. Hence, laboratory conformation is necessary during early stage infection [2,9]. Every case detected is treated as follows [5,15]: Nothing can be done until the worm begins to ulcerate the skin. Once the worm begins to emerge, the affected part is dipped in clean water. The extruding worm is then gradually rolled by a stick and properly bandaged, and the patient received adequate health education not entering a water source. Pain killers such as aspirin are given to help reduce pain and inflammation. Other control and eradication strategies include, access to an improved drinking water supply which is imperative for eradication [13]. Improved drinking water sources can make a difference in guinea worm disease affected communities [2,5,20].

There are several ways to improve the drinking water such as the construction of barriers to prevent humans from entering surface drinking water, protecting hand dug wells with walls and sinking deep bore wells, and finally filtering or boiling surface drinking water through sieves [13]. Vector control, by treating the contaminated stagnant drinking water sources with the larvicide, Tempos. Tempos application to unsafe stagnant drinking water sources is effective at killing the copepods but must be initiated within 14 days of case detection [2]. Finally, community based health education and mobilization for increased adherence to control interventions is necessary for control and strategic eradication of Guinea worm disease [5]. The overarching goal of the health education and social mobilization is to encourage affected communities to adopt healthy behavior to prevent and ultimately interrupt the disease transmission [2].

Challenges

The current challenge to eradication of guinea worm disease are to interrupt the transmission cycle in the few last foci of the four remaining countries and ensure surveillance in all other countries at risk of disease re-introduction [1]. Although there is need for continued nationwide surveillance and awareness of new cases, it is important that a diagnostic assay be developed to evaluate future cases as well as to monitor re-emergence potential [2]. Currently, it is not yet known if other species of copepod have similar reactions inside human bodies once ingested [16], this could affect the outcome of infection from them to human host, additionally, it is not well understood how the adult female worm manages to evade the human immune system for over a year without discomfort or inflammatory response so an early diagnostic assay would prove most effective in validating and locating these female worms before they begin to cause debilitating disease as well as rule out other types of parasitic infections [2,16].

Grant Proposal Objective

It is my objective that a diagnostic assay for determining early onset of D. medinensis infection can be developed and work effectively in a timely manner to use in countries were guinea worm disease is still endemic in anticipation of future vaccine development and ultimately eradication of the disease. It is my aim that use of the VHH camelid antibodies can be used to develop a diagnostic assay for early detection of Dracunculiasis infection [21,22]. This is relevant because antigenicity prediction from D. medinensis can

play an important role in prototype synthetic vaccine development as well as target validation. My three specific aims for addressing the need for a diagnostic assay are outlined as follows.

Specific Aim 1: Determine an appropriate antigen from D. medinensis to effectively use for the assay.

Rational for Specific Aim 1: The rational for this specific aim is to predict secreted protein antigens released by the worm that will be identified in the serum of infected patients using Western blot and proteomic analysis. These antigens are typically composed of either peptides or polysaccharides and bind to antigen specific receptors in the host serum [21]. These antigens that are recognized by antibodies that can be used as a guide in vaccine components design and immuno- diagnostic reagents. The purpose of the need of VHH antibodies to bind to female worms is that, after the mating process male worms quickly die out. It is the female that persists a year henceforth growing and migrating around the body before exiting to lay her eggs [12].

Specific Aim 2: The second aim will be to validate a VHH Ab to use as platforms that will bind to secreted antigen of adult female Guinea worms.

Rational for Specific Aim 2: The second aim to prove my hypothesis is to validate a VHH antibody to use as a platform for the assay by determination of viability and functionality of multiple secreted proteins. VHH antibodies are heavy chain only single domain molecules produced by camelids [21,22]. Their small size (~15kDa) enables them to penetrate the blood brain barrier, and be expressed in *E coli*. These molecules have the ability to recognize unique conformational epitopes with the dominant involvement of its ling complementary determining [12] region 3 [21,22]. VHH's are temperature resistant, and have high stability making them a good tool to use for diagnostic purposes. In addition, they possess the ability for high specificity and affinity. The aim is to identify viable antigens from aim #1 then obtain the coding DNA in an expression vector. This will be used to immunize Alpaca's for a course of 3 months, after which time blood will be collected to isolate serum allowing VHH's can be screened to recognize the antigen of Guinea worms [4,12]. Finally, the VHH's will be expressed in phage *E.coli*, purified, cloned, and later tested in Aim #3. The use of these VHH's is expected to give high yields.

Specific Aim 3: The third aim of developing a diagnostic assay for early parasitic infection is to include both positive and negative controls for the assay.

Rational for Specific Aim 3: The third aim to achieve my hypothesis is to have a positive and negative control for validity of assay quality assurance of functionality. The VHH's purified in aim #2 will now be tested for their ability to detect the target antigen in patient serum. There are three groups allocated to this testing [4]. The first being actively infected individuals, the second being post patent infections, and the third group has never been afflicted with GWD. Then we will test the ability of these VHH's to correctly identity patients who have known GWD as positive controls, while also using patients in non-endemic areas as negative controls [12].

Antibody isotype	*Dracunculus medinensis* infection status[a,b]		
	Group 1 (patent)	Group 2 (post-patent)	Group 3 (controls)
Total IgG			
Plasma	1.44 (1.3–1.7)**	1.33 (0.9–1.6)**	0.89 (0.6–1.2)
Tear fluid	1.65 (0.5–1.9)**	0.18 (0–1.8)	0.21 (0–1.6)
IgG_1			
Plasma	1.15 (1.1–1.3)**	0.54 (0.3–0.8)**	0.39 (0.1–0.5)
Tear fluid	0.1 (0–1.3)**	0 (0–0.4)	0 (0–0.3)
IgG_3			
Plasma	0.67 (0.4–1.8)*	0.33 (0.1–1.6)	0.15 (0.1–0.9)
Tear fluid	0 (0–1.3)	0	0
IgG_4			
Plasma	0.49 (0.1–2.0)**	0.96 (0–2.0)**	0.03 (0–0.5)
Tear fluid	0.16 (0–2.0)**	0 (0–1.9)	0 (0–0.8)

[a] Antibody reactivity (OD at 405 nm) is shown as median OD and range (in parentheses).
[b] Significant differences between patient groups and controls are indicated as ** $P \leq 0.01$ or * $P < 0.05$.

Table 1: Assay relating DmAg- specific reactivity of IgG1, IgG3, IgG4, and total IgG is shown in patent, post patent, and control groups.

Expected Outcome

This assay is expected to do three things, have a quick turnaround time, be accurate, and detect early infections. From this assay I plan to learn if the VHH's are able to make a strong binding affinity with the D. medinensis surface protein epitopes in a timely and accurate fashion [21].

Potential problems and alternative strategies

Potential challenges of this assay remain the fact that immunological responses of humans to D. medinensis remain inconclusive potentially jeopardizing the accuracy of the assay [17]. Also, funding for camelids to be properly immunized, housed, and a steady supply of samples to be obtained for analysis and transports to India, Asia, and Sub-Saharan Africa without denaturing, thawing or incorrectly running the assay [19,21,22]. In addition, due to the fact that this disease is so close to eradication it may be difficult to find and obtain funding from the NIH or any institution to develop the materials required [3]. Additionally, there are no animal models in which to test this assay other than humans. Other possible explanations for any negative results may be that the secreted products from the Guinea worms are not antigenic, not specific, or were discharged in quantities too low to measure, or were degraded in the host tissues and rapidly removed from circulation [4].

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