IMPACT OF THE ENVIRONMENT ON DEVELOPMENT OF HELMINTH AND PROTOZOAN INVASIVE ELEMENTS AND CONTEMPORARY METHODS FOR SANITARY-PARASITOLOGICAL DIAGNOSTICS: REVIEW

Mihaela Videnova

National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria, Department of Parasitology and Tropical Medicine

ABSTRACT

According to the World Health Organisation, more than 1.5 billion of the world population is affected by parasitic diseases caused by geohelminths. The number of persons suffering from foodborne and waterborne protozoan diseases is similar. In developed countries, including Bulgaria, systematic sanitary and parasitological studies of soil and water are the basis for monitoring and control in protecting public health. Occurrence and prevalence of human parasitic infections is determined by the peculiarities of life cycle of parasites, peculiarities of hosts as reservoir sources, the abiotic and biotic factors of the environment as a complex providing conditions for the development or sterilisation of different parasite stages, as well as socioeconomic factors that play a leading role in the whole epidemiological process.

Systematic sanitary-parasitological studies require the application of classical and novel reliable, sensitive and practical diagnostic methods that are also easy to perform, economical and efficient enough.

KEYWORDS:

sanitary parasitology, factors, methods

ADDRESS FOR CORRESPONDENCE:

Mihaela Videnova
Bulgaria, 1504 Sofia,
26 Yanko Sakazov Blvd., National Centre of Infectious and Parasitic Diseases,
Department of Parasitology
and Tropical Medicine;
email: mvidenova@ncipd.org; Phone: +35929446999; ext. 316 /Fax: +35928438002

Sources of parasitic diseases are infected humans or animals, where the parasites produce invasive elements. The power of the source is determined by the amount of infectious material excreted per day in the external environment, and depends on the type of parasite and its reproductive capacity.

The mechanism of parasite transmission and penetration is specific and is accomplished through a number of biotic, abiotic and social factors that provide the opportunity of the invasive forms to enter the host body. Certain features of the host, e.g. resistance, immunity, age, etc., as well as those of propagative stage of the parasite, clarify the role of biological factors in the epidemic process of parasitic diseases. The mechanisms of dissemination, transmission and penetration of invasive stages in the macroorganism of the healthy and susceptible human population, under the specific economic, cultural and household conditions, are the social factors in the epidemic process. These two types of factors (biological and social) determine the course of the epidemic process (1).

In some cases the release of parasites into the environment occurs through faeces, urine, sputum, vaginal discharge (anal-oral and urogenital anthroponotic diseases). Important for their subsequent distribution is either entering the irrigation systems, drinking water sources or contamination of ground-growing vegetables, fruits, household objects, hands, food, etc. Other parasites circulate in the blood and lymph fluid of hosts or are found in their muscles, internal organs, or skin (2).

The most commonly reported routes for transmission of infection, particularly in humans, are as follows (3):

- Ingestion of eggs, cysts, larvae or hosts with invasive stages of the parasites (*Entamoeba* spp., *Giardia* spp., *Ascaris* spp., *Toxocara* spp., *Enterobius*

vermicularis, *Trichuris* spp., *Echinococcus* spp., *Blastocystis* spp., *Trichinella* spp.)
- Inoculation of invasive stages by host or vector (malaria plasmodia, leishmania, filaria).
- Direct penetration of invasive stages of parasites into the human body through the skin (*Ancylostoma* spp., *Schistosoma* spp., *Strongyloides* spp.)

ABIOTIC AND BIOTIC FACTORS INFLUENCING THE DEVELOPMENT AND SURVIVAL

Soil conditions are an important factor for the egg development rate. Main limiting factors are temperature and soil moisture. Eggs and other invasive stages of helminths have different survival time in the soil. For example, the eggs of *Ascaris lumbricoides*, *Teania saginata,* and *Trichuris trichiurus* can survive in soil for several months, while cysts of *Entamoeba histolytica* - less than 10 days.

Survival of parasites outside the host`s body depends on environmental conditions. The eggshell is one of the most resistant biological structures that offer a high degree of protection for the developing embryo. It is highly impermeable and only lipid solvents, gases, and water molecules pass through it (4).

Factors influencing the survival of cysts of parasitic protozoa and helminth eggs and larvae can be grouped into three categories:
- Physical factors - temperature, sunlight, ultraviolet radiation, etc.
- Chemical factors - oxygen concentration, acidity (pH) of the soil, various chemicals, etc.
- Biological factors - pathogenic and ovicidal fungi, protozoa, invertebrates, etc.

Temperature is one of the physical factors mainly affecting the development of invasive elements, such as size, maturation, survival and infectivity of the free parasite stages. At high temperatures, nematode larvae slow down their function, fall into heat stupor, and die above 60°C. Some geohelminths avoid these unfavourable for their development conditions by transition from one stage to another; different species can tolerate different temperature range (8). Under pasteurisation conditions (90°C), high temperature is used for the remediation of sludge from invasive eggs of *Ascaris* spp. Eggs of *Taenia saginata* lose viability in five minutes at 71°C and the eggs of *Necator americanus* in 50 minutes at 45°C (9).

On the other hand, low temperatures (8.9°C to 15.6°C) prevent the full embryonation of the egg. Under experimental conditions *Ascaris* spp. eggs are stored in a refrigerator at temperature of about 7-8°C. In some parasitic species low environmental temperatures can have a limiting effect, while in others they have no significant influence. For example, the 3rd-stage larvae of *Trichostrongylidae* species survive at temperatures below -28°C in pastures in Poland (5).

A high percentage of lost viability is observed when eggs and partially developed embryos of *Trichiuris* spp. are exposed to very low temperatures (-9°C to -12°C) (6). The larvae release is sharply reduced after prolonged exposure (from 77% to 47%). The *Ascaris suum* eggs remain viable for a period of 40 days when they are exposed to temperatures ranging from -18°C to -27°C (7).

Another important factor for the survival of parasites and their invasive elements is light and ultraviolet radiation. Both factors have a direct impact on some of the stages in the life cycle of parasitic nematodes (10). It is believed that the colouring of eggshells of certain zooparasitic nematodes is associated with additional protection in relation to ultraviolet radiation.

Eggs of *Trichiurus* spp. are significantly more resistant to the impact of light. It is supposed that the dark pigmentation of their eggshell protects them from the shorter wave lengths of ultraviolet light. For example, even very short exposure of *Ascaris* spp. eggs is sufficient for disturbances of embryonic development to occur, and longer is lethal (6).

Humidity of the soil is a major factor for the survival of helminths and many of them practically are unable to survive in the dry soil substrate. It is of great importance whether soil drought occurs slow or fast. For example, *Trichostrongilus colubriformis* can survive 164 days when drought occurs slowly within 3-4 days (11). Eggs of some zooparasitic nematodes lose their water very slowly when subjected to drying and this allows the larva to

survive water stress. However the eggs of *Ascaris* spp. survive at low and zero humidity levels only up to 3 days regardless of temperature (7). The optimal conditions for their normal development are humidity about 80% and temperature above 20°C.

EFFECTS OF DRYING ON THE VIABILITY OF PARASITE EGGS IN SLUDGE

There is a correlation between eggs viability and moisture content of the sludge. Eggs inactivation increases with decrease in moisture in the sludge from the drying layer. The lowest humidity levels at which all eggs of *Ascaris* spp. and *Toxocara* spp. become inactive are 5% in autumn, 7% in winter, 8% in spring and 15% in summer. It has been found that both temperature and decrease in moisture content play a role in the inactivation of these parasites. Drying the sludge on air to very low humidity levels leads to complete destruction of the parasites present there.

Another group of factors affecting the survival of the parasites and their elements involved in invasion are the chemical ones. The most important from these factors are soil oxygen concentration and pH.

The lack of oxygen suppresses the overall metabolism of many nematodes and affects their development and survival. For *Ascaris* spp. eggs the development rate sharply decreases under low oxygen concentration as they are obligate aerobes (12). Non-embryonated eggs can survive for several weeks at room temperature under anaerobic conditions but their growth is inhibited. In nature eggs of *Ascaris* spp. can adapt to developing in a low-oxygen environment. Adult helminths inhabiting the human intestinal tract live in practically anaerobic conditions and have primary anaerobic metabolism (13, 14). However, their eggs require mandatory oxygen exposure to continue their normal development.

ACIDITY (PH)

Parasite eggs are considered to be very resistant to extreme pH values (15). The optimal pH for the development of *Necator americanus* eggs is about 6. The ecological significance of this is that the faeces and soil provide optimal pH for hatching as well as contain the necessary nutrients and electrolytes for further development of the larvae to the invasive stage (15).

The eggs of *Ascaris* spp. can be embryonated in a wide range of relatively toxic solutions such as 14% hydrochloric acid, 9% sulfuric acid, 8% acetic acid, 0.4% nitric acid, 0.3% carboxylic acid, 0.5% sodium hydroxide, 1% mercuric chloride, 4% formaldehyde as well as in a number of chemicals used for cleaning and disinfection. Concentrations of ozone and chlorine at levels of 4.0 and 40 mg/l, respectively, were found to destroy the eggs of *Shistosoma mansonia* while ozone has no effect on the eggs of *Ascaris* spp. and *Hymenolepis* spp. (16). The resistance of these eggs to toxic substances is due mainly to the structure of the inner membrane of the eggshell, which is lipoid in nature (17).

BIOTIC FACTORS

The development of parasitic eggs and protozoan cysts also depends on different biological factors. Soil pathogenic fungi are capable of attacking and destroying eggs of *Ascaris lumbricoides* (18). Under experimental conditions this occurs for several days or weeks. The rate of the ovicidal effect depends on the type of ovicidal fungi and the active substances they release. The parasitic fungus *Cylindrocarpon radicola* penetrates and destroys the helminth eggs (19, 20). A number of invertebrates, especially insects and snails, can also mechanically kill the eggs of helminths. It has been found that between 10% and 20% of the eggs of *Ascaris* spp. are discharged from *Planorbis planorbis, P. corneus, Bithynia tentaculata*, structurally damaged and incapable of further development. From 8% to 10% of the eggs develop only to the gastrula. The embryogenesis of the remaining eggs is delayed by 10 to 15 days (21).

ASSESSMENT OF THE VIABILITY OF HELMINTH EGGS AND LARVAE

Determination of the viability and number of discovered eggs/larvae for each sample is necessary and important in order to carry out risk assessment in accordance with certain international and national standards and guidelines (22, 23).

The most widely used method of assessing the

viability is incubation. Solutions of sulfuric acid (24, 25) and formalin (26) are used to incubate the isolated eggs at incubation temperatures ranging between 22°C and 26°C and duration of the incubation period between 21 and 30 days (26). The solution of sulfuric acid (0.1N) was reported to give the best result, between 75-80% (22) and 83-92% (27) viability, followed by formalin solution (75-80%) (26). The disadvantage of incubation in determining the viability of helminth eggs is the duration of time. The morphological integrity of the eggs and their response to dye staining, as well as their characteristic features as size, shape, and presence of visible larvae are used as a criterion for the viability of the eggs.

This is avoided by the use of dyes differentiating viable from non-viable eggs based on the permeability of the eggshells. The most widely used in practice are the Lugol solution (28), Safranin O (24) and Eosin Y (29). When comparing the results using different dyes versus conventional incubation, was found that conventional incubation detected 86% of viable eggs, which was lower than the viable eggs determined by staining with safran (97%), crystal violet (92%) and methylene blue (87%). The lowest viability (39%) was reported for trypan blue staining (30). Disadvantage of the colouring is that some dyes are toxic to the embryos, so the sample testing should be done within a few minutes after the application of the dye (31).

CLASSICAL METHODS FOR DETECTION AND IDENTIFICATION

Most of the methods used for detection and grading of external environment samples for the presence of helminth eggs, larvae and protozoan cysts are performed by standard light microscopy and are called conventional methods. They include sedimentation followed by an extraction stage and then flotation prior to microscopy (32). However, all these steps may vary depending on the type of the sample, its amount, pretreatment of the sample and the type of searched parasites and their different stages that can be identified in the environment.

Samples from different sources and their quantity are distinguished in the individual test matrices – sewage, sludge, compost, soil. Detection of helminth eggs and protozoan cysts is hampered by their uneven distribution in the environment. This feature can be compensated by the collection of a large number of single samples from a given habitat and their homogenisation. This method is used more often in samples of sludge, compost, faeces (26).

In addition, eggs of *Ascaris* spp., *Trichuris* spp., and *Toxocara* spp. are highly resistant to degradation of sediments and the removal or even the inactivation of the parasites requires subsequent disinfection steps. However, some of them maintain their invasive potential even after severe treatment conditions. For example, Maya et al. (24) assessed the degree of inactivation of *Ascaris lumbricoides, Ascaris suum, Toxocara canis,* and *Trichuris trichiura* eggs when subjected to 80°C and pH 12.1 and found that less than 25% of these parasites were inactivated under these conditions.

An important step is the concentration of samples and the separation of helminth eggs and protozoan cysts from sludge containing multiple compacted particles. This is often done by sedimentation, which may be passive, or by using a different speed centrifugation. So far, however, there is no commonly accepted methodology by the different laboratories in the world (33).

A critical step during separation of the eggs from the rest of the particles is flotation. Different solutions such as $MgSO_4$, $ZnSO_4$, NaCl, $NaNO_3$ and sucrose solutions are used, but a saturated solution of NaCl is the most widely used for flotation.

CURRENT METHODS OF DIAGNOSIS

PCR techniques are emerging as very specific, sensitive and rapid methods for detection of different pathogens in different matrices of waste water to soil and food products (34, 35). Among the PCR methods developed for detecting pathogens are the quantitative polymerase chain reaction (qPCR), multiplex polymerase chain reaction (mPCR) and drip-digital polymerase chain reaction (ddPCR) (36). One of the main obstacles to the efficient detection of eggs by using molecular methods is the extraction of a nucleic acid with good quality

and quantity, which is hampered by their hard shell (37). Furthermore, the presence of large amounts of suspended solids in the samples also hinder the extraction of nucleic acid (38) and can inhibit the PCR reactions. Separation of eggs from these solid particles is carried out prior to extraction of DNA (38) using flotation and/or sedimentation steps. The ability to analyse species-specific gene sequences by PCR makes the method preferred in cases of identification difficulties with standard morphological methods (39). Several other studies have shown that qPCR is much more sensitive than conventional microscopic methods for detecting helminths in different environmental samples (40).

In Bulgaria sanitary-parasitological surveys are carried out by the Regional Health Inspectorates (RHI). Environmental investigations for the presence of parasites and their invasive elements are conducted annually and results are announced in the annual reports. For the period 1995-2009 were examined 6414 samples of soil from settlement foci of soil-transmitted helminthiasis, sandboxes in childcare facilities and parks. In 54 samples (0.84%) were found eggs of *A. lumbricoides*, *T. trichiuris* and larvae of *Trichostrongylidae* spp. (41). The relative share of positive samples shows a sustainability trend from 0.52% (1996) to 0.54% (2008). Several other studies have been published in recent years by Georgieva et al, 1999 and 2005 (42, 43), Muhtarov, 2016 (44) and Popova, 2018 (45). In samples from playgrounds, gardens, and garbage bins in Stara Zagora, the authors found *Toxocara* spp. eggs in 17 samples, *Trichiurus* spp. eggs in 5 samples, *Taenia* spp. eggs in 4 samples, and *Dipilydium caninum* in 3 samples (42, 43). A total of 301 samples from environment were investigated in Kardzhali district and in 71 (23.59%) were found parasitic invasive forms, from which about 1% were eggs of *Taenia* spp. (44). Similar results were obtained in a sanitary-parasitological study conducted in Plovdiv region, where about 2% of the samples were contaminated with eggs of *Taenia* spp. (45).

For the period from August to October 2017, in the National Reference Laboratory for Diagnosis of Parasitic Diseases at the NCIPD were tested 180 samples of soil, sand, open water sources and sludge from wastewater treatment plants. Conventional (microscopic) and biomolecular (PCR) diagnostic methods were used. In the studied samples of different substrates with microscopic analysis were found 16 (9%) positive: 5 positive for nematode larvae, 2 positive for eggs of *Ancylostoma* spp. and 9 positive specimens for oocysts of *Cryptosporidium* spp. By using PCR methods, the number of positive samples increased to 22 (11%): 4 positive for oocysts of *Toxoplasma gondii*, 1 positive for oocysts of *Cryptosporidium* spp., 1 positive for *Toxocara canis* eggs (46). In this respect, our data on sensitivity of PCR techniques is similar to data found in the literature. Furthermore, biomolecular methods allow species identification with high accuracy including specimens that are unfit for morphological analysis or are morphologically identical (e.g. *Taenia* spp. eggs).

In conclusion, we can state that in order to perform quality sanitary-parasitological studies it is necessary to include the biomolecular methods for species identification of the detected helminth eggs and protozoan cysts. This would significantly improve the quality of the research on environmental samples for the presence of invasive parasitic elements as well as the measures for surveillance and control of parasitic diseases.

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