Report of STSM at the European Reference Laboratory for Parasites, Istituto Superiore di Sanità, Rome, Italy

Title of the STSM Mission "Tracking the genetic diversity of *Giardia* Isolates by molecular biology and bioinformatics"

By

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Giardia duodenalis is a common protozoan parasite responsible for enteric illness in humans and animals worldwide. Human transmission is predominantly through the fecal–oral route (person-to-person) and contaminated water (drinking and recreational). However, there has also been considerable interest and discussion surrounding the potential for zoonotic transmission of these pathogens, particularly from livestock [1,2,3,4,5,6,7]. Transmission of *Giardia* may occur through either direct contact in the case of farmers, veterinarians, and petting zoos, or through indirect routes such as contaminated surface water or food [8].

The prevalence and concentration of *Giardia* cysts in raw wastewater depend on several factors such as sanitation conditions, socioeconomic status, levels of annual precipitation and on the sensitivity of the detection methods. *Giardia* cysts have been reported to be prevalent at higher levels in raw wastewater samples analyzed in various parts of the world (9, 10). *Giardia* cysts were detected in almost 100% of the analyzed raw wastewater samples in various countries at a concentration range of 2 to 13,600 cysts/L and no substantial differences were

observed in the concentration of *Giardia* cysts in raw wastewater from various countries (11).

A considerable amount of data has shown that *Giardia* should be considered as a species complex that comprises at least eight distinct genetic groups, referred to assemblages A to H (12, 13, 14). To date, assemblages A and B have been mostly associated with human infections (15). However, genetic traits that influence the virulence and other aspects of the infection are unknown and efforts to correlate the parasite genetic make-up and the clinical symptoms in the host have generated controversial results (15).

Three different *G. duodenalis* Assemblages have been reported in cattle. Assemblage A, which has a wide host range including humans, and livestock-specific Assemblage E, are frequently recognized in cattle. Assemblage B, which represents the second *G. duodenalis* Assemblage found in humans, also has a wide host range, but has only more recently been identified in cattle (16,17). Numerous studies have reported a higher prevalence of Assemblage E in cattle than Assemblage A (4), although Uehlinger et al. (2006) reported comparable prevalence rates for both Assemblages in adult dairy cattle, suggesting a greater risk of zoonotic transmission than previously thought (18). Geurden et al. (2008) reported a higher prevalence of Assemblage A than E in dairy calves in Belgium, while Assemblage E predominated in beef calves [17]. Similarly, Santin et al. (2009) demonstrated that infections with the zoonotic Assemblage A are more common in dairy calves than previously reported, suggesting that calves may be an important source of human infection [18]. Data regarding the zoonotic potential of giardiasis is lacking in Israel.

Therefore, in order to apply effective measures to protect public health from contracting giardiasis, it is important to determine the origin of *Giardia* circulating in the environment. Therefore, the objective of this STSM was to introduce Dr. Nasser to molecular tools useful to determine the origin of the parasites in clinical and environmental samples. The laboratory of Dr. S. M. Cacciò was chosen because of the will established methodology for assemblage classification of *Giardia* parasites by various techniques such as PCR-RFLP and sequence analyses.

The specific aims are

1. Genotyping of *Giardia* by conventional molecular biology methods such as PCR-RFLP.
2. Application of sequencing to determine molecular variability of *Giardia* isolates of clinical and environmental origin.
3. Tracking the source of *Giardia* species detected in environmental samples by sequencing, to determine zoonotic sources of transmission.

The following methods and techniques were introduced:

1. Detection of *Giardia* cysts from stool samples by Immuno-Fluorescent (IF) staining and enumeration by Epi-Fluorescent microscope.
2. Polymerase Chain Reaction (PCR) for the detection of *Giardia* cysts from stool and environmental samples. The detection of *Giardia* by PCR consists of the optimal primer sequences of specific genes and the PCR conditions.

3. Amplification of the primary PCR band by secondary PCR. This method increases the sensitivity of the PCR method, which makes the PCR suitable for the detection of low level of *Giardia* cysts from environmental and clinical samples.

4. Digestion of fragments generated by nested PCR, by using restriction enzymes, to distinguish between the different *Giardia* assemblages in clinical and environmental samples. Fragments generated by restriction enzyme digestion were visualized by capillary gel electrophoresis, and the profile analyzed to determine the Assemblage in each sample. Difficulties in determining the Assemblages were discussed in relation to similarity of restriction profiles.

5. Introduction to sequence analysis of *Giardia* sequences generated following nested PCR. Techniques to edit Sanger sequences were introduced. BLAST searches were used to determine the level of homology with all sequences present in the GenBank database, and to assign isolates at the level of Assemblage and sub-Assemblage.

Results generated by STSM

1. Detection of *Giardia* Cysts by IF and Epi-Fluorescent microscope

Staining of *Giardia* cysts in stool samples by monoclonal antibodies labeled with FITC, and observation of the cysts by epi-fluorescent microscope (Figure 1). The staining was performed according to manufacturer's instruction (Meridian).

Attention was given to the morphology (size, shape, staining intensity) of the cysts.

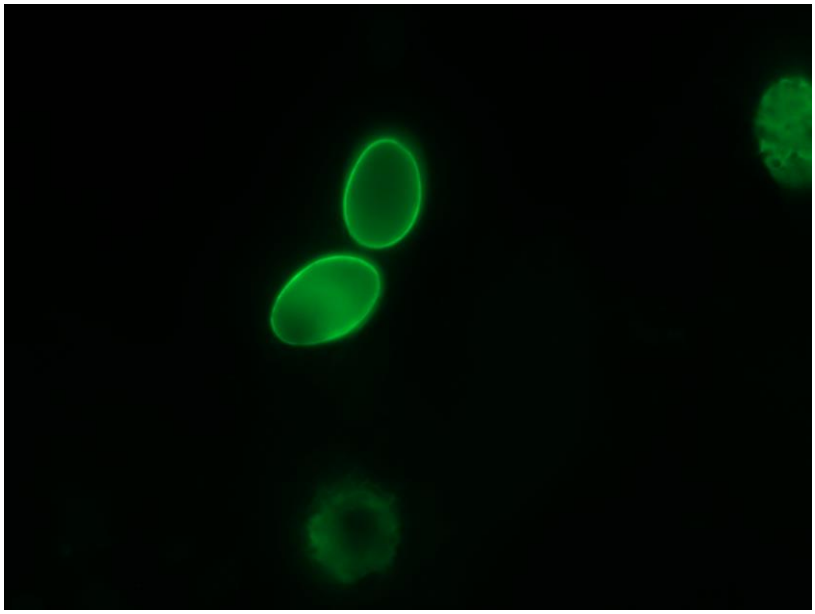

Figure 1. *Giardia* cysts stained with FITC labeled monoclonal antibodies and visualized in an epi-fluorescent microscope at 100X.

2. DNA purification from stool samples positive for *Giardia* cysts using the FastPrep® FP120 Instrument and the Fast DNA spin kit for soil. The methods allow isolating DNA from stool or soil samples in less than 30 minutes. DNA was isolated from three stool samples positive for *Giardia* cysts. Extracted DNA was subsequently used for nested PCR experiments.

3. Nested PCR for the detection of a β-giardin gene fragment.

   The results are presented in Figure 2. Although most of the samples were negative by primary PCR, seven out of the 10 samples were positive by

nested PCR, indicating that nested PCR increases the sensitivity of detection.

PCR program used: 94°C x 3’ (94°C x 30’’-55°C x 30’’-72°C x 1 min.) for 35 cycles -72° C x 7’, gene B-giardin; amplicon size 723 bp.

Nested PCR program used: 94°C x 3’ (94°C x 30’’-53°C x 30’’-72°C x 1 min.) for 35 cycles -72°C x 7’, gene B-giardin; amplicon size 511 bp.

Primers for primary PCR for B-giardin

G7 [5′-A AGCCCGACGACCTCACCCGCAGTGC-3′] and

G759 [5′-GAGGCCGCCCTGGATCT TCGAGACGAC-3′].

Primers for secondary PCR for B-giardin

F Nbg [5′-CTCGACGAGCTT CGTGTT-3′] and

R Nbg [5′-GAACGAACGAGATCGAGGTCCG-3′]

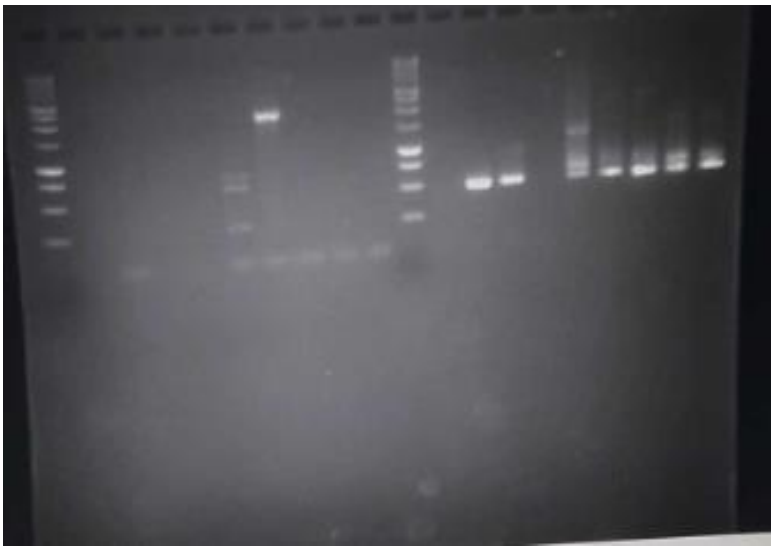

Figure 2. Agarose gel electrophoresis of PCR and nested PCR products of the *Giardia* β-giardin gene; amplicon size: 723 bp for the first PCR, and 511 bp for the nested PCR.

4. Digestion of the nested PCR products by Hae III restriction enzyme (PCR-RFLP) to identify *Giardia* at the level of Assemblage.

The method is based on the amplification of a 511 bp fragment by PCR and nested PCR, followed by digestion of the fragment by the endonuclease HaeIII, which allows the identification of assemblages according to restriction profiles. The visualization of the restriction products was accomplished by capillary gel electrophoresis (Figure 3). The results presented in Figure 3 indicate that five out of the 10 (50%) analyzed samples were identified as assemblage A, three out of 10 (30%) as assemblage F, 1 out of 10 (10%) as assemblage B and 1 out of 10 (10%)

as assemblage D. The results demonstrated that PCR-RFLP allows the identification of the *Giardia* Assemblages present in stool samples.

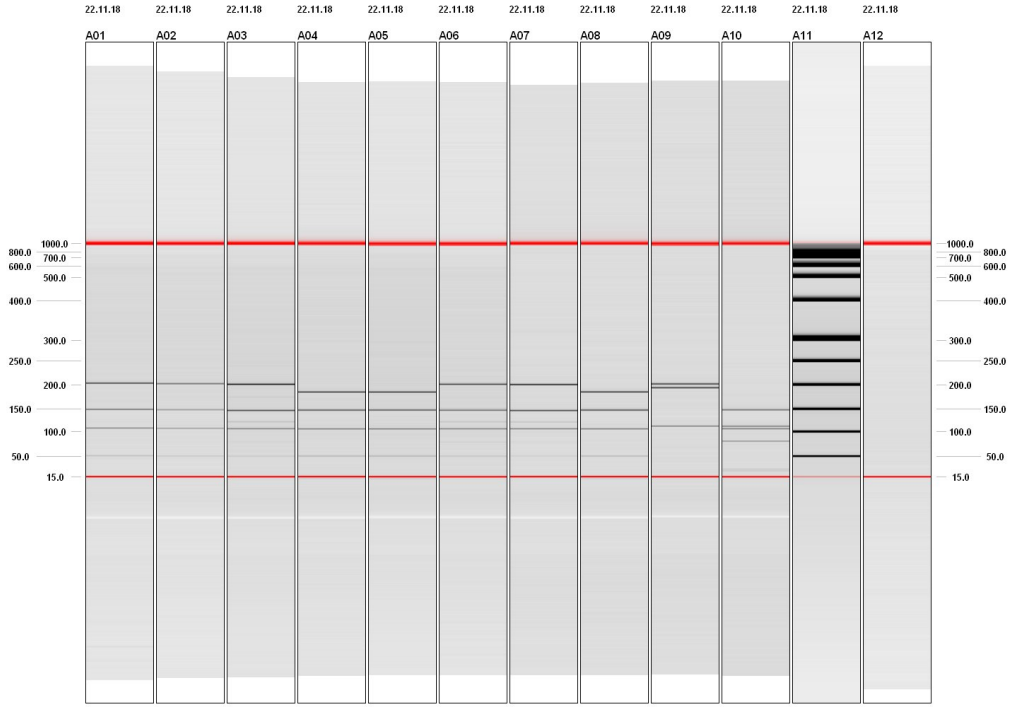

Figure 3. Capillary Gel Electrophoresis of HaeIII restriction fragments of nested PCR products of the beta giardin gene from various stool samples.

5. Sequence analysis to identify Assemblages and sub-genotypes of *Giardia*

During my stay, I was introduced to sequence analysis, which consists of Sanger sequencing of purified nested PCR fragments. The sequencing reaction can be outsourced to a company, which provides this service upon request. The sequences are then carefully edited, and compared, using BLAST searches, to all

homologous sequences stored in the GenBank database. Three exercises were performed to demonstrate identification of *Giardia* at the sub-genotype level.

Conclusions

1. Standardized methods for DNA extraction from environmental and stool samples were demonstrated.
2. The feasibility of detecting low levels of *Giardia* by PCR and nested PCR was demonstrated.
3. The suitability of PCR-RFLP to identify *Giardia* cysts at the assemblage level was demonstrated using the capillary electrophoresis for sensitive and specific fragment analysis.
4. The suitability of sequence analysis to identify *Giardia* isolates at the level of sub-genotypes was demonstrated.

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