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BRIEF COMMUNICATION

COMPARISON BETWEEN IMMUNOMAGNETIC SEPARATION, COUPLED WITH IMMUNOFLUORESCENCE, AND THE TECHNIQUES OF FAUST *et al*. AND OF LUTZ FOR THE DIAGNOSIS OF *Giardia lamblia* CYSTS IN HUMAN FECES

Doris Sobral Marques SOUZA(1), Juliana Teixeira BARREIROS(2), Karina Maria PAPP(2), Mário STEINDEL(3), Cláudia Maria Oliveira SIMÕES(4) & Célia Regina Monte BARARDI(3)

SUMMARY

In the present study, the performance of Immunomagnetic Separation technique, coupled with Immunofluorescence (IMS-IFA), was compared with the FAUST *et al*. and Lutz parasitological techniques for the detection of *Giardia lamblia* cysts in human feces.

One hundred and twenty-seven samples were evaluated by the three techniques at the same time showing a rate of cyst detection of 27.5% by IMS-IFA and 15.7% by both Faust *et al*. and Lutz techniques. Data analysis showed a higher sensitivity of IMS-IFA for the detection of *G. lamblia* cysts in comparison with the techniques of FAUST *et al*. and Lutz.

The use of this methodology as a routine procedure enables the processing of many samples simultaneously, in order to increase recovery rate of *G. lamblia* cysts and reduce the time of sample storage.

KEYWORDS: *Giardia lamblia;* Giardiasis; *Cryptosporidium;* Immunomagnetic separation technique; Diagnosis.

Giardia is a flagellated intestinal protozoan parasite, responsible for causing disease in various animal species including humans. Considered the most common intestinal pathogen worldwide[7], *Giardia lamblia* (syn.: *Giardia intestinalis* and *Lamblia intestinalis*) presents two forms: trophozoite and cyst during its biological cycle. *Giardia* infection occurs by the fecal-oral route through the ingestion of water or food contaminated with cysts[4]. The general estimated prevalence of giardiasis among children in Brazil is 28.5% in various geographic areas[1]; in some environments, such as nurseries schools, orphanages, hospital wards, etc, its prevalence can be still higher[7]. In both symptomatic and asymptomatic carriers cyst elimination is not continuous, but may last for long periods[7].

Laboratory diagnosis for *Giardia* is based on stool processing by different techniques and light microscope examination. Examination of fresh diarrheal specimens is useful for trophozoite detection whereas the centrifuge-flotation methods (Faust *et al*., Sheather, etc.) or centrifuge-sedimentation (Ritchie) are more adequate for cyst detection. The smears can be stained by Lugol's iodine or iron hematoxylin[2]. Antibody and antigen detection, by Enzyme-Linked Immunosorbent Assay (ELISA), is also used for diagnosis in humans, as well as the detection of the parasites in water samples[10].

Immunomagnetic Separation coupled with Immunofluorescence (IMS-IFA), is a technique based on the use of magnetic beads conjugated to specific monoclonal antibodies against the cell surface markers of bacteria, protozoa, etc. Immunomagnetic separation accomplishes the isolation of these specific structures from their environment: water, feces, mud, etc, while IFA can confirm the identification of the isolated structures[3,8,9,14]. The IMS-IFA technique has been used in tests for *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in environmental samples in our laboratory[5]. This technique was previously standardized for *Giardia lamblia* and *Cryptosporidium* spp. diagnosis in human feces. This was performed by seeding the feces with an internal sample control, that consisted of *C. parvum* oocysts and *G. lamblia* cysts (99 ± 1 of each) labeled with Texas Red® (ColorSeed®, BTF Pty Ltd., Australia). This procedure allows artificially seeded oocysts and cysts to be distinguished, from those belonging to the sample. Since no significant differences between the recovery average of the two parasites in chi-square analysis[11] were observed, the final option was to use an "external control" that consisted of a suspension of *Cryptosporidium parvum* oocysts (donated by Dr. Duncan Veal from Macquarie University, NSW, Australia – 10^5 oocysts/mL), prepared in the laboratory, as this was less expensive than the former.

(1) Mestranda do Programa de Pós-Graduação em Farmácia da Universidade Federal de Santa Catarina (UFSC), SC, Brasil.
(2) Acadêmicas da 6ª Fase da Graduação em Medicina da UFSC, SC, Brasil.
(3) Laboratório de Virologia Aplicada MIP/CCB. UFSC, SC, Brasil.
(4) Departamento de Ciências Farmacêuticas CIF/CCS, SC, Brasil.
Correspondence to: Drª Célia R.M. Barardi, Laboratório de Virologia Aplicada, MIP, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, SC, Brasil. E-mail: ccb1crb@ccb.ufsc.br. Phone: +55.48.331-5207, Fax: + 55.48.331-9258.

The aim of this study was to compare the IMS-IFA technique with Faust *et al*. and Lutz techniques[2] for the detection of *G. lamblia* cysts in human feces. This study received approval from the Ethical Committee of Universidade Federal de Santa Catarina, SC (Nº 005/2001).

One hundred and twenty-seven stool samples were obtained from children (one per child), between 2 and 12 years old, from a nursery school, Alfa Gente Vila Aparecida (77 samples), and from two hospitals, Hospital Infantil and Hospital Universitário da Universidade Federal de Santa Catarina (50 samples), in Florianópolis, State of Santa Catarina. Feces were fixed in formalin-sodium acetate-acetic acid (SAF) in a proportion of 1:5 feces/SAF and stored at room temperature (20 °C) for no longer than 4 months. Stool samples were processed by Faust *et al*. and Lutz methods[2], by three students under the supervision of the responsible teacher, for cysts detection using standard protocols according to DE CARLI and by IMS-IFA technique as described by GREINERT (2001).

The magnetic beads (BIOMAG® - Polyscience Inc, Warrington, USA) used in this study were composed of approximately 1 µm (diameter) magnetic particles consisting of an iron oxide core with a silane coating. They are also commercially available with goat anti-mouse IgG covalently attached and were conjugated with specific monoclonal antibodies (Mab) at the laboratory. Briefly, magnetic beads were washed twice in 6 mL tubes in Bead Buffer (PBS added with 1% (w/v) BSA, 0.1% (w/v) SDS and 0.05% (w/v) Sodium Azide). The beads were incubated with hybridoma supernatants, for 16 hours in an orbital shaker (Mini-rotator, Glass-Col®) at 4 °C. These hybridoma supernatants contained Mab, raised against either the walls of *Giardia* spp. cysts (Mab G203) or the walls of *Cryptosporidium* spp. oocysts (CRY104). Mab G203 and CRY104 were generously donated by Dr. Duncan Veal from Environmental Pathogens Lab, Macquarie University, NSW, Australia (3.0 mL of cell supernatants and 3.0 mL of Bead Buffer). Afterwards, the tubes were placed in an adequate rack, containing a magnet. The supernatant was discarded and the beads were resuspended in 6.0 mL of Bead Buffer and stored at 4 °C.

For IMS-IFA assay (that was performed by one person, but the membrane examination under the microscope was performed by two different people) 1 mL of formalin-fixed human stool was suspended in two centrifuge tubes (tubes 1 and 2) containing 7 mL of Bead Buffer. In one of the tubes (tube 2), 10 µL of the standard *Cryptosporidium* oocyst suspension was added (positive-control). Tubes were vortexed for 30 sec and left on the bench for 40 min for spontaneous sedimentation. Thereafter, each supernatant was transferred to a 6 mL capacity tube, and then 200 µL of Magnetic Beads, coupled with G203 (tube 1) or CRY104 (tube 2), was added. The suspensions were incubated in an orbital shaker, for 30 minutes, and transferred to the magnet-containing rack, for bead separation during 15 minutes. These supernatants were transferred to two fresh tubes (1' and 2') and returned to the magnet-containing rack to ensure that all the magnetic beads were captured. After the two-step capture procedure and supernatant discarding, the beads were washed in 4 mL of Bead Buffer, resuspended in 4 mL of 0.1M glycine buffer, pH 2.2, and vortexed for two minutes. The suspension was agitated in orbital shaker for 15 min (acid dissociation), and finally placed on the magnetic shelf for Magnetic Bead capture for five minutes. Each supernatant containing either cysts or oocysts was filtrated through two 8 µm Nucleopore® Membranes, (Whatman, New Jersey, USA) and the cysts or oocysts incubated for 5 min with 100 µL of anti-mouse IgG coupled with FITC (Sigma Inc, USA) 1:100 dilution in Bead Buffer (Fig. 1).

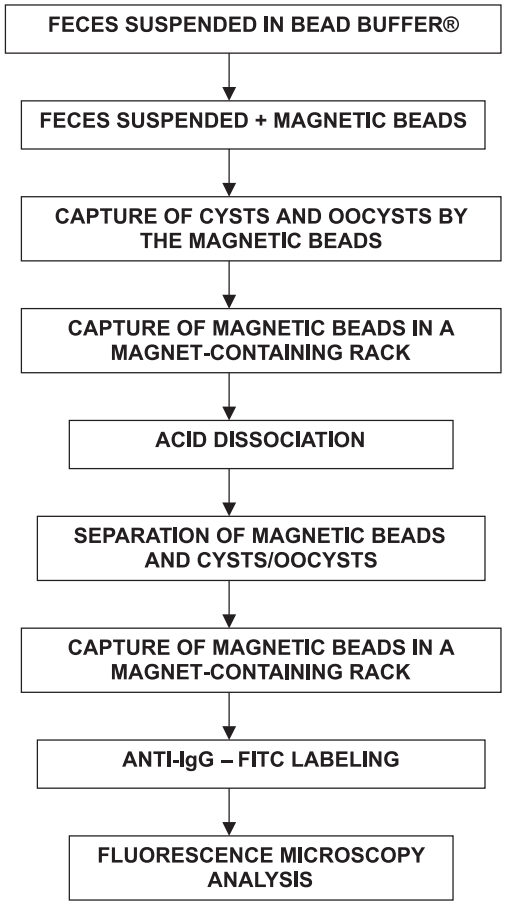

Fig. 1 - Processing of human stool samples by Immunomagnetic separation, coupled with Immunofluorescence.

The membranes were covered with a slide glass and examined in a BX-40 FLA (Olympus®) fluorescence microscope (200 X and 400 X) for the presence of *Giardia* cysts *and Cryptosporidium* oocysts. For the seeded samples, the number of oocysts was counted in order to calculate the cyst recovery rate (Fig. 2). Data were analyzed by the Chi-square test (χ^2).

IMS-IFA analysis of the 127 processed stool samples resulted in 35 (27.6%) positive samples of *G. lamblia* cysts and 92 negative samples. Using standard Faust *et al.* and Lutz methods[2], 20 samples (15.7%) were positive and 107 were negative. Twenty out of the 35 positive IMS-IFA samples, were confirmed by both Faust *et al*. and Lutz methods[2] (Table 1).

IMS-IFA proved to be a very sensitive and specific technique for *G. lamblia* cyst detection when compared with the standard Faust *et al*. and Lutz methods[2] ($\chi^2 = 5.22$, GL = 1, $p < 0.05$). In addition, the labeled cysts are very bright, turning their visualization easier thereby avoiding misinterpretations, since some artifacts can interfere in the correct

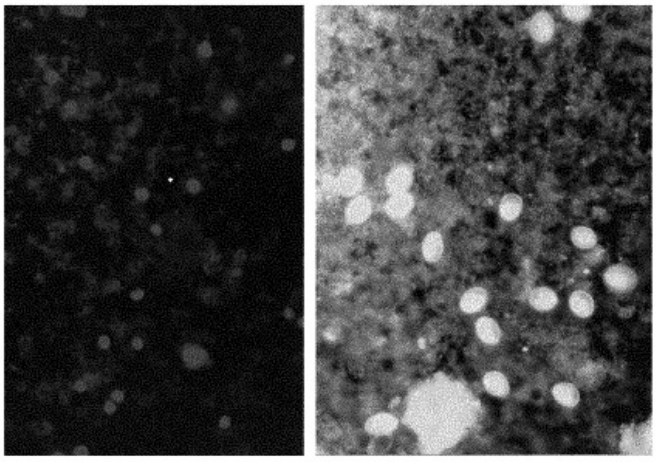

Fig. 2 - Detection of oocysts of *Cryptosporidium* spp. **(left)** and cysts of *Giardia lamblia* **(right)** by Immunofluorescent Microscopy (FITC), 400X, in human feces.

Table 1
Detection of *Giardia lamblia* cysts in human feces by Immunomagnetic Separation, coupled with Immunofluorescence (IMS-IFA), and Faust *et al.* and Lutz techniques

Techniques	Number	Positive Samples n (%)	Negative Samples n (%)
IMS-IFA	127	35 (27.6%)	92 (72.4%)
Faust *et al.*	127	20 (15.7%)	107 (84.3%)
Lutz	127	20 (15.7%)	107 (84.3%)

evaluation because the cysts are small and the amount of debris is usually large. This paper reports for the first time the use of IMS-IFA technique for the detection of *G. lamblia* cysts or *Cryptosporidium* oocysts in human feces. IMS-IFA has been successfully used to detect *Cryptosporidium* spp. oocysts in both environmental samples and bovine feces[5,8,9,14]. Since the standardization of this methodology for human stool samples, in our laboratory, showed no differences in the rates of either cyst or oocyst recovery, positive controls were seeded only with *Cryptosporidium* oocysts.

According to WEBER *et al.*[12], the limit of oocyst recovery in seeded human stool was 10^4 oocysts/mL using the formalin-ethyl-acetate technique even in diarrheic stool samples, where the oocyst count is usually higher. The threshold of *Cryptosporidium* spp. oocyst recovery in seeded bovine feces was 10^4 oocysts/g of feces using the Ritchie technique and 4 x 10^3 oocysts/g of feces for the Sheater method[13]. On the other hand, in calf feces (which are more fatty than adult stool) seeded with 2 x 10^3 oocysts/g of feces, the average rate for oocyst recovery was 3.6 ± 1.0 oocysts using the Faust method[6]. During the standardization of IMS-IFA methodology for human stool in our laboratory, seeded samples with 10^2 and 10^3 oocysts/g reached a rate of oocyst recovery of 4.7% and 8.5% respectively[11]. These results were 20% better than the oocyst recovery from calf stool using the Faust *et al*. method, as reported by KUCZYNSKA & SHELTON[6].

Although IMS-IFA was shown to be more sensitive than Faust *et al*.[2] and Lutz methods, it is more costly. Consequently it is necessary to evaluate the advantage of its use and find ways of reducing costs. The use of this methodology as a routine procedure enables the processing of many samples simultaneously, in order to increase recoveries and reduce the time of sample storage[6].

RESUMO

Comparação entre a imuno-separação magnética, acoplada à imunofluorescência, e as técnicas de Faust *et al*. e de Lutz para o diagnóstico de cistos de *Giardia lamblia* em fezes humanas

No presente trabalho, o desempenho da técnica de Imunoseparação Magnética, acoplada à Imunofluorescência (IMS-IFA), foi comparado com aqueles das técnicas parasitológicas de FAUST *et al*. e de Lutz na detecção de cistos *de Giardia lamblia* em fezes humanas. Foram processadas 127 amostras de fezes pelas três técnicas paralelamente e a detecção de cistos foi de 27,5% para IMS-IFA e de 15,7% para as técnicas de FAUST *et al*. e de Lutz concomitantemente. A análise dos resultados mostrou maior sensibilidade da IMS-IFA na detecção de cistos de *G. lamblia* quando comparada aos métodos de FAUST *et al*. e Lutz.

A utilização desta metodologia como procedimento de rotina proporciona o processamento de várias amostras simultaneamente, além de aumentar a recuperação de cistos de *G. lamblia* e reduzir o tempo de estocagem das amostras.

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REFERENCES

1. CIMERMAN, B. & CIMERMAN, S. - **Parasitologia humana e seus fundamentos**. São Paulo, Atheneu, 1999.

2. DE CARLI, G.A. - **Diagnóstico laboratorial das parasitoses humanas. Métodos e técnicas.** Rio de Janeiro, Medsi, 1994.

3. DI GIOVANNI, G.D.; HASHEMI, F.H.; SHAW, N.J. *et al*. - Detection of infectious *Cryptosporidium parvum* oocysts in surface and filter backwash water samples by immunomagnetic separation and integrated cell culture-PCR. **Appl. environ. Microbiol., 65**: 3427-3432, 1999.

4. DORSCH, M.R. & VEAL, D.A. - Oligonucleotide probes for specific detection of *Giardia lamblia* cysts by fluorescent *in situ* hybridization. **J. appl. Microbiol., 90**: 836-842, 2001.

5. GREINERT, J.A. - **Avaliação de técnicas para detecção e quantificação de *Giardia* spp. e *Cryptosporidium* spp. em água de piscina**. Florianópolis, 2001. (Dissertação de Mestrado - Centro de Ciências Biológicas da Universidade Federal de Santa Catarina).

6. KUCZYNSKA, E. & SHELTON, D.R. - Method for detection and enumeration of *Cryptosporidium parvum* oocysts in feces, manures and soils. **Appl. environ. Microbiol., 65**: 2820-2826, 1999.

7. NEWMAN, R.D.; MOORE, S.R.; LIMA, A.A.M. *et al*. - A longitudinal study of *Giardia lamblia* infection in North-East Brazilian children. **Trop. Med. Int. Hlth.**, **6**: 624-634, 2001.

8. PEREIRA, M.D.C.; ATWILL, E.R. & JONES, T. - Comparison of sensitivity of immunofluorescent microscopy to that of a combination of immunofluorescent microscopy and immunomagnetic separation for detection of *Cryptosporidium parvum* oocysts in adult bovine feces. **Appl. environ. Microbiol., 65**: 3236-3239, 1999.

9. ROCHELLE, P.A.; DE LEON, R.; JOHNSON, A.; STEWART, M.H. & WOLFE, R.L. - Evaluation of immunomagnetic separation for recovery of infectious *Cryptosporidium parvum* oocysts from environmental samples **Appl. environ. Microbiol.**, **65**: 841-845, 1999.

10. SOGAYAR, M.I.T.L. & GUIMARÃES, S. - *Giardia lamblia*. In: NEVES, D.P.; MELO, A.L.; GENARO, O. & GINARD, P.M., ed. **Parasitologia humana**. 10. ed. São Paulo, Atheneu, 2000. p. 107-113.

11. SOUZA, D.S.M. - **Avaliação de técnicas de diagnóstico de *Cryptosporidium* spp. e *Giardia lamblia* em fezes humanas**. Florianópolis, 2002. (Dissertação de Mestrado - Centro de Ciência da Saúde da Universidade Federal de Santa Catarina).

12. WEBER, R.L; BRYAN, R.T.; BISHOP, H.S. *et al*. - Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: evidence for low sensitivity of current diagnostic methods. **J. clin. Microbiol.**, **29**: 1323-1327, 1991.

13. WEBSTER, K.A.; SMITH, H.V.; GILES, M.; DAWSON, L. & ROBERTSON, L.J. - Detection of *Cryptosporidium parvum* oocysts in faeces: comparison of conventional coproscopical methods and the polymerase chain reaction. **Vet. Parasit.**, **61**: 5-13, 1996.

14. YAKUB, G.P. & STADTERMAN-KNAUER, K.L. - Evaluation of immunomagnetic separation for recovery of *Cryptosporidium parvum* and *Giardia duodenalis* from high-iron matrices. **Appl. environ. Microbiol.**, **66**: 3628-3631, 2000.

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