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Short Communication **Open Access**

Improved *Cryptosporidium* case findings using immunofluorescent microscopy on concentrated stool

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Abstract:

Background: Diarrhoea is a major cause of morbidity in Cape Town, South Africa, and mortality is attributed to a failure to recognize the severity of the condition. *Cryptosporidium* and *Giardia* are increasingly recognized as important causes of diarrhoea in Africa however, suboptimal diagnostic techniques may lead to underappreciation of their significance. Our objectives are to compare the diagnostic yield of direct immunofluorescent antigen (DFA) microscopy on concentrated stool samples for *Cryptosporidium* and *Giardia*, with the current approach of wet mount microscopy for *Giardia* and auramine fluorescent stain for *Cryptosporidium* on unconcentrated stool.
Methodology: Stool specimens (n=104) received at our hospital laboratory for routine microbiological investigations were used for the study. Direct wet-mount auramine-phenol fluorescent microscopy (auramine) detection of *Cryptosporidium* oocysts and wet mount iodine microscopy for *Giardia* detection, were performed on unconcentrated stool samples, while DFA stain for simultaneous detection of *Cryptosporidium* and *Giardia* was performed on sodium-acetate formalin concentrated stool samples. The diagnostic yields of the tests were compared using the MEDCALC® version 18.0
Results: Of the 104 stool specimens received for microbiological analysis, only 66 (63.5%) had specific *Cryptosporidium* requests while 38 (36.5%) had no *Cryptosporidium* specific requests. Of the 66 specimens, 9 (13.6%) were positive for *Cryptosporidium* oocysts with DFA while only 1 (1.5%) was positive with auramine staining (p=0.013). The one auramine-positive specimen was also positive by DFA. Auramine stain microscopy gave a sensitivity of 11.1% (95%CI: 0.28-48.25%) and specificity of 100% (95%CI: 93.7%-100%) when compared to DFA. Of the 38 stool specimens without specific *Cryptosporidium* request, DFA yielded 5 (13.2%) additional positive results. Taken together, *Cryptosporidium* was detected in 14/104 (13.5%; 95%CI: 8.36–21.7%) specimens and only 1 of 14 (7.1%) specimens with the current routine laboratory testing approach. *Giardia* was detected by DFA in 3/104 (0.9%) specimens, while direct iodine wet mount microscopy did not yield any positive results (0%). All 3 *Giardia*-positive specimens had *Cryptosporidium* oocysts detected by DFA.
Conclusion: These data suggest that a large proportion of *Cryptosporidium* cases remain undetected by the laboratory due to suboptimal testing methods, and failure by clinicians to specifically request for *Cryptosporidium* detection. There is need to periodically assess the effectiveness of diagnostic microbiology laboratory approaches to diarrhoea, and access to improved diagnostic laboratory techniques will contribute to more accurate differential diagnosis and a broadened understanding of local aetiology of diarrhoea diseases in Africa.

Keywords: *Cryptosporidium*, *Giardia*, diarrhoea, stool concentration, DFA, microscopy

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Amélioration des découvertes de cas de *Cryptosporidium* à l'aide de la microscopie immunofluorescente sur des selles concentrées

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Abstrait:

Contexte: La diarrhée est une cause majeure de morbidité au Cap, en Afrique du Sud, et la mortalité est attribuée à l'incapacité de reconnaître la gravité de la maladie. *Cryptosporidium* et *Giardia* sont de plus en plus reconnus

comme des causes importantes de diarrhée en Afrique, cependant, des techniques de diagnostic sous-optimales peuvent conduire à une sous-estimation de leur importance. Nos objectifs sont de comparer le rendement diagnostique de la microscopie à antigène immunofluorescent direct (DFA) sur des échantillons de selles concentrées pour *Cryptosporidium* et *Giardia*, avec l'approche actuelle de la microscopie à montage humide pour Giardia et la coloration fluorescente auramine pour *Cryptosporidium* sur des selles non concentrées.
Méthodologie: Des échantillons de selles (n=104) reçus au laboratoire de notre hôpital pour des examens microbiologiques de routine ont été utilisés pour l'étude. La détection directe par microscopie fluorescente auramine-phénol à montage humide (auramine) des oocystes de *Cryptosporidium* et la microscopie à l'iode à montage humide pour la détection de *Giardia*, ont été effectuées sur des échantillons de selles non concentrées, tandis que la coloration DFA pour la détection simultanée de *Cryptosporidium* et de Giardia a été réalisée sur de l'acétate de sodium formaline concentré échantillons de selles. Les rendements diagnostiques des tests ont été comparés à l'aide de MEDCALC® version 18.0
Résultats: Sur les 104 échantillons de selles reçus pour l'analyse microbiologique, seuls 66 (63,5%) avaient des demandes spécifiques de *Cryptosporidium* tandis que 38 (36,5%) n'avaient pas de demandes spécifiques de *Cryptosporidium*. Sur les 66 échantillons, 9 (13,6%) étaient positifs pour les oocystes de *Cryptosporidium* avec DFA tandis que seulement 1 (1,5%) était positif avec coloration à l'auramine (p=0,013). Le seul échantillon positif à l'auramine était également positif au DFA. La microscopie à l'auramine a donné une sensibilité de 11,1% (IC 95%: 0,28-48,25%) et une spécificité de 100% (IC 95%: 93,7% -100%) par rapport au DFA. Sur les 38 échantillons de selles sans demande spécifique de *Cryptosporidium*, le DFA a donné 5 (13,2%) résultats positifs supplémentaires. Pris ensemble, *Cryptosporidium* a été détecté dans 14/104 (13,5%; IC à 95%: 8,36–21,7%) et seulement 1 des 14 échantillons (7,1%) avec l'approche actuelle des tests de routine en laboratoire. *Giardia* a été détecté par DFA dans 3/104 (0,9%) échantillons, tandis que la microscopie directe à l'iode sur monture humide n'a donné aucun résultat positif (0%). Les 3 échantillons positifs à *Giardia* avaient des oocystes de *Cryptosporidium* détectés par DFA.
Conclusion: Ces données suggèrent qu'une grande proportion des cas de *Cryptosporidium* ne sont pas détectés par le laboratoire en raison de méthodes de test sous-optimales et de l'échec des cliniciens à demander spécifiquement la détection de *Cryptosporidium*. Il est nécessaire d'évaluer périodiquement l'efficacité des approches de laboratoire de microbiologie diagnostique pour la diarrhée, et l'accès à des techniques de laboratoire de diagnostic améliorées contribuera à un diagnostic différentiel plus précis et à une compréhension élargie de l'étiologie locale des maladies diarrhéiques en Afrique.

Mots clés: *Cryptosporidium, Giardia*, diarrhée, concentration des selles, DFA, microscopie

Introduction:

Diarrheal disease in developing countries account for up to 21% of deaths in children less than 5 years. Approximately 78% of these occur in Africa and South East Asia (1). In South Africa, diarrhea is the third leading cause of infant mortality (16%), trailing behind deaths in the neonatal period (27.5%) and HIV/AIDS (19.5%) (2). In the Western Cape Province, diarrhoea is the third leading cause of under-five mortality (11%); almost half (42.9%) of child diarrheal deaths in the Cape Town metro sub-district occur at home (3). Among those who seek primary healthcare, some are locally managed, while others are referred to tertiary care; a main contributing factor for diarrheal death among those referred to tertiary care in South Africa is a failure to correctly assess the severity of the child's condition (4).

The enteric coccidian parasites, *Cryptosporidium parvum* and *Cryptosporidium hominis*, and the protozoan parasite, *Giardia duodenalis* are important causes of diarrhea globally. *Cryptosporidium* is increasingly recognized as a leading cause of moderate-to-severe diarrhea in both immunocompetent and immunosuppressed subjects (5). The Global Enteric Multicenter Study (GEMS) showed that *Cryptosporidium* is second only to Rotavirus as a contributor to moderate-to-severe diarrhea in sub-Saharan Africa (6). *Cryptosporidium* is transmitted via contaminated food, water, and from person-to-person particularly where suboptimal sanitation and limited access to safe drinking water prevail. Similarly transmitted, *Giardia* is a major cause of intestinal disease globally, with a higher prevalence in Africa (7). Chronic infection can lead to weight loss and malabsorption and is associated with stunting, wasting and cognitive impairment in children (8).

Clinical management guidelines highlight the need, importance and calls for improved effectiveness of test and treat approaches to diarrhoea (1,9,10). Availability of accurate diagnostic laboratory tests are neglected, and evaluation and improvements are much needed (1,11,12). Our objectives were to employ stool concentration and immunofluorescent stain for the detection of *Cryptosporidium* and *Giardia* on all stool samples received for microbiological investigations at an academic pathology laboratory in Cape Town, South Africa, and compare findings to those obtained with the current methods employed by the laboratory.

Materials and method:

Study setting and specimens

Unpreserved diarrheal stool specimens (n=104) received for routine microbiological investigations at the National Health Laboratory Services (NHLS), University of Cape Town Department of Pathology at Groote Schuur Hospital, were collected from 12 June through

6 August 2014. The NHLS laboratory receives specimens for testing from hospitalized patients as well as specimens from secondary hospitals and clinics in the Southern Cape Town area, including the Red Cross Children's Hospital, a national paediatric referral hospital. Current routine laboratory investigations include direct wet mount with iodine microscopic analysis of stool specimens. Auramine-phenol fluorescent microscopy is performed when *Cryptosporidium* or *Isospora* testing is specifically requested. The remains of stool specimens after routine testing were collected by the researchers who were blinded to testing results.

Ethical approval

This study was approved by the Human Research Ethics Committee, Faculty of Health Sciences, University of Cape Town (Ref: 240/2014).

Microscopic examination of specimens

A grape sized amount, or a 5ml aliquot of liquid stool, was added to 40ml of sodium acetate-acetic acid-formalin (SAF) in a 50ml conical centrifuge tube and mixed by inversion until homogenous mixture was attained. The preserved stool samples were concentrated using a commercial kit (Para-Pak® Spin-Con® Stool Concentration System, Meridian Bioscience, Cincinnati, OH) according to the manufacturer's package insert (13). A direct immunofluorescent assay (DFA) (Merifluor® *Cryptosporidium/Giardia* Direct Immunofluorescent Assay, Meridian Bioscience) was performed according to the manufacturer's instructions (14).

Each slide was viewed under an LED fluorescent microscope with an excitation wavelength of 490-500nm under the 40x objective lens with a calibrated eyepiece 10x ocular lens with micrometer (total magnification 400x) for specific size measurements. Samples were screened for *Cryptosporidium* oocysts and *Giardia* cysts based on colour, shape and size. Both internal kit controls and external control slides were included in each staining process. External control slides comprising stool specimens previously shown to contain *Cryptosporidium* oocysts and *Giardia* cysts were kindly provided by the Centre for Opportunistic, Hospital and Tropical Diseases, National Institute for Communicable Diseases, South Africa.

Statistical analysis

Statistical calculations for diagnostic test evaluation were performed using MEDCALC® version 18 (www.medcalc.org) and McNemar's test for significance was applied. P value less than 0.05 was considered to be statistically significant.

Results:

Of the 104 stool specimens received for microbiological analysis, only 66 (63.5%) had specific *Cryptosporidium* request. Of the 66 stool specimens tested for *Cryptosporidium* oocysts using both the routine direct wet-mount auramine-phenol fluorescent microscopy and the DFA microscopy on concentrated specimen, only 1 (1.5%) specimen yielded a positive auramine finding compared to 9 (13.6%) with DFA (p=0.013). The one auramine-positive specimen was also positive by DFA. Direct wet-mount auramine microscopy yielded a sensitivity of 11.1% (95% CI: 0.28%-48.25%) and specificity of 100% (95% CI: 93.7%-100%) when compared to DFA.

Of additional 38 stool specimens received for microbiological analysis without a specific request for *Cryptosporidium* testing (no direct auramine), DFA yielded 5 (13.2%) additional positive results. Taken together, *Cryptosporidium* was detected in 14/104 (13.5%; 95%CI: 8.36–21.7%) specimens, and only 1 of 14 (7.1%) cases was successfully detected with the current routine laboratory testing approach. *Giardia* was detected by DFA in 3/104 (0.9%) specimens, while direct iodine wet mount microscopy did not yield any positive results (0%). All 3 *Giardia*-positive specimens had *Cryptosporidium* oocysts detected in them by DFA.

Discussion:

Case findings of *Cryptosporidium* in concentrated stool combined with DFA microscopy was significantly higher (13.6%) than those obtained using unconcentrated stool microscopy with auramine (1.5%). Similarly, case findings of *Giardia* were enhanced with DFA on concentrated stool (4.5%) compared to direct iodine microscopy (0%). An additional 5 of 38 cases of *Cryptosporidium* were detected in stool without a request for *Cryptosporidium* investigation. These data suggest that a large proportion of *Cryptosporidium* cases remain undetected by the laboratory due to suboptimal testing methods used, as well as a failure by clinicians to specifically request testing for *Cryptosporidium*.

The *Cryptosporidium* prevalence of 13.5 % (14/104) reported in this study is in line with findings elsewhere in South Africa. In the Northern metro district of Cape Town Nel et al., reported 10.4% of children under 5 years of age with diarrhoea yielded *Cryptosporidium* (15). In Limpopo province, *Cryptosporidium* was reported in 25.2% of hospital patients and 17.9% of diarrheal samples in school children (16). Our findings are consistent with the recognized laboratory standards

recommending concentration of stool for recovery of intestinal parasites, and the improved sensitivity and specificity of DFA for the detection of *Cryptosporidium* (17,18).

This study was limited to the months of June to August representing the winter/rain season in the Western Cape. The etiological causes of diarrhea may be affected by seasonality. However, *Cryptosporidium* is more commonly found in cases of diarrhoea than is suspected. Relying on unconcentrated stool for detection of *Cryptosporidium* is therefore suboptimal. DFA detection on concentrated stool improves case findings. These findings suggest the need to periodically assess the effectiveness of the microbiological diagnostic approach into the causes of diarrhoea.

Authors contributions:

FJLR conceptualized the study, obtained funding, and provided supervision. DC performed the investigation and curated the data. Both authors contributed to the analysis of data and writing of the manuscript.

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Conflicts of interest:

No conflicts of interest are declared.

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