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MODIFIED KÖSTER STAINING METHOD FOR THE DIAGNOSIS OF CRYPTOSPORIDIOSIS

by

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Summary — This paper describes a modified Köster staining method for the specific coprodiagnosis of cryptosporidiosis in human and animal faeces and the efficacy of this technique is compared with the other staining methods currently in use. Both the modified Köster and Ziehl Neelsen techniques are reliable and provide a clear distinction between cryptosporidian oocysts and yeasts although the former technique gives a better differentiation of the internal structures of cryptosporidia.

KEYWORDS : *Cryptosporidium*; Cryptosporidiosis, Diagnosis; Modified Köster Staining.

Introduction

The genus *Cryptosporidium* belongs to the family Cryptosporiidae, order Eucoccidiida, class Sporozoa, Phylum Apicomplexa and is found in the intestinal tract of man and several species of animals and occasionally in the respiratory system of birds (Soulsby, 1982; Anderson, 1982).

For a long time it was considered an opportunistic infection. Although the pathogenesis of cryptosporidiosis is not fully understood, its pathogenicity under experimental and natural conditions is now clearly demonstrated (Tzipori, Angus & Campbell, 1982; Pohlenz *et al.*, 1978).

Cryptosporidia usually affect animals of younger age group; usually between few days after birth and 4 weeks of age. This increased susceptibility of younger animals is probably the result of a subdued immunocompetence because of the immature immunological system of the host. Immunodeficient animals infected with *Cryptosporidium* sp. are shown to suffer from persistent diarrhoea (Tzipori, 1983).

In humans with a normal functioning immune system, cryptosporidiosis causes a moderate and usually self-limiting enterocolitis (Current *et al*,. 1983; Tzipori, 1983; Jokipii, Pomyola & Jokipii, 1983). However, a severe and persistent diarrhoea lasting a few months to even years has been reported in patients with Acquired Immune Deficiency Syndrome (AIDS) (Ma and Soave, 1983; Current *et al.*, 1983; Andreani *et al.*, 1983), with congenital immunodeficiencies (Lasser, Lewin & Tying, 1979; Sloper *et al.*, 1982) and in patients receiving immunosuppressive therapy following transplantation (Meisel *et al.*, 1979).

The present paper describes the modified Köster method for the staining of *Cryptosporidium* sp. and emphazises its relevance for the routine parasitological diagnosis of cryptosporidiosis.

Materials and Methods

Materials

Four known positive faecal samples from calves with neonatal diarrhoea due to cryptosporidiosis were used as reference. These samples were kindly provided by the « Laboratoire de Virologie, Centre d'Economie Rurale », Marloie, Belgium.

Faecal samples of human and animal origin are routinely examined for *Cryptosporidium* sp. at the Veterinary Department, Institute of Tropical Medicine. A sample originating from a patient with AIDS was submitted for examination and found positive for cryptosporidiosis. It was the first case of human cryptosporidiosis demonstrated in Belgium. Samples from this patient were examined every week for one month and this demonstrated the persistence of the infection. Subsequently, 20 more faecal samples of calves with neonatal diarrhoea from Brabant (Belgium) were examinated and two out of these were found positive for *Cryptosporidium* sp.

To check the specificity of the staining method, faecal samples, either from humans or from calves were mixed with cryptosporidia as well as yeasts from laboratory cultures (*Cryptococcus albidus, Candida albicans, Torulopsis glabrata*) and processed as described. Faecal specimens without cryptosporidia were treated in a similar way.

Techniques

a) *Concentration methods :*

Both, SAEX (Loughlin & Spitz, 1949) and Sheather's sugar solution methods were used. The latter was slightly modified (Antoine, 1982 pers. comm.) as following. To about 5 g of feaces 15-20 ml PBS was added, mixed and filtered. The filtrate was centrifuged at 500 g for 10 minutes. After another washing in PBS, the sediment was mixed with a sucrose solution (45 g in 355 ml distilled water), centrifuged at 500 g for 10 minutes, and finally a loopful from the surface of the supernatant was placed on a slide and examined.

b) *Staining techniques :*

Thin faecal smears were prepared, either directly or after concentration of the faecal sample. For the latter a small quantity of plasma or albumin was added to support the faecal material on the slides. The smears were dried at room temperature or on a hot plate at 37 °C if plasma or albumin was incorporated. Smears were also stained by Ziehl-Neelsen, Giemsa or May-Grünwald methods as recommended by Hendriksen and Pohlenz, 1981; Pohlenz *et al.*, 1978; Peeters *et al.*, 1982 respectively. The results of these staining techniques were compared with the modified Köster staining method given below, mainly based on the procedure of Alton, Jones & Pietz, 1977.

The smears were fixed in methyl alcohol for 2 to 5 minutes and the slide held over a Bunsen flame for a short time to dry. They were stained for 5 minutes in a mixture containing 2 parts of an aqueous saturated

solution of safranin and 5 parts of an aqueous solution of potassium hydroxide (5,6 per cent). The slides were rinsed with water and differentiated in a 0.1 per cent solution of sulphuric acid for 10 seconds, then rinsed again with water and finally counterstained with an aqueous solution of malachite green (5 per cent) for 10 to 15 seconds, excess of malachite green was washed off with water. The slides were dried and examined under the microscope using the oil immersion objectives. When compared with the original procedure (Alton *et al.*, 1977), the procedure of fixation, the time of staining with safranin and the concentration of differentiation fluid are modified in this method. Besides, malachite green rather than methylene blue is preferred for counter-staining.

In addition to these staining methods for permanent preparations, the carbol fuchsin method (Heine, 1982) was also used as quick staining method whereby the entire examination has to be done within 10 minutes.

Results and Discussion

Several methods are described to detect and identify *Cryptosporidium* (Ma & Siave, 1983; Garcia *et al.*, 1983). Until recently the most reliable method of diagnosis in human medicine was by scanning or transmission electron microscopy of intestinal biopsy materials and demonstrating the cryptosporidia at different stages of development on the brush border of the intestinal epithelial cells. Nevertheless, collection of such biopsy material and electron microscopy are difficult procedures for routine laboratory use. Faecal concentration followed by the use of a staining technique appears to be the most appropriate alternative for reliable diagnosis of cryptosporidiosis.

The procedure described in this paper stains the 3 to 5 μm cryptosporidia pale red against a greenish background. The oocysts exhibit internal structural details which are probably representing the sporozoites, this is in contrast to the modified Ziehl-Neelsen technique of Hendriksen and Pohlenz, 1981. No other organisms including yeasts found in faecal material show staining characteristics similar to those of *Cryptosporidium*. Furthermore no differences in morphological or staining affinity between the cryptosporidia of human or animal origin were observed with this procedure.

Several useful faecal concentration methods have been described (Iseki, 1979; Anderson, 1983; Willson & Acres, 1982). In our observations both the SAEX and Sheather's sugar solution methods were satisfactory and showed similar results. Nevertheless, some faecal organisms which are comparable in size and morphology to cryptosporidia make exact diagnosis difficult. Therefore the above described staining methods are recommended to improve the accuracy of the parasitological diagnosis.

Recently, the authors demonstrated the first human case of cryptosporidiosis in Belgium in a patient suffering from AIDS. Comparing the different available staining methods it was found that the modified Köster staining is the most accurate method to detect and identify faecal *Cryptosporidium* of human or animal origin.

The original Köster's staining procedure (Alton, Jonas & Pietz, 1977) is a selective method used for specific demonstration of *Brucella*. Provided

some modifications on the fixation, the timing of the first staining, the differentiation period and the choice of counterstain, the Köster staining method appears to be specific for *Cryptosporidium.* No other material in faecal samples shows an identical staining affinity and structural details.

The specificity of staining was confirmed when yeasts were added to the faecal samples of human or animal origin positive for *Cryptosporidium.* A clear distinction between *Cryptosporidium* and yeasts can be made both by Köster and Ziehl Neelsen modified methods, whereas with the Giemsa staining method the distinction is not always easy. It is noted that the temporary staining with carbol fuchsin is an excellent screening method (Heine, 1982).

In conclusion, we found the modified Köster and Ziehl Neelsen techniques quite reliable for the diagnosis of cryptosporidiosis in faecal samples due to their staining specificity for the oocysts, but the Köster's method had an added advantage because of a shorter staining time and a better differentiation of the internal structural details of the cryptosporidian oocysts.

Smears of positive faecal samples stained with the modified Köster method as well as the transparencies of the preparation are available on request at the Veterinary Department, Institute of Tropical Medicine, Antwerp, Belgium.

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Gewijzigde Köster kleuring voor de diagnose van cryptosporidiosis.

Samenvatting — Deze mededeling beschrijft een gewijzigde Köster-kleuring, specifiek voor het aantonen van *Cryptosporidium* in faeces van menselijke of dierlijke oorsprong. Tevens wordt de doeltreffendheid van bewuste methode vergeleken met andere algemeen toegepaste kleurmethoden. De gewijzigde Köster- en Ziehl Neelsen-kleuring zijn beide betrouwbaar en maken een duidelijk onderscheid tussen gisten en oocysten van *Cryptosporidium* mogelijk, alhoewel de eerste techniek een betere differentiatie van de strukturen der cryptosporidia toelaat.

La coloration de Köster modifiée dans le diagnostic de la cryptosporidiose.

Résumé — La présente communication décrit une technique de coloration, la technique de Köster modifiée, comme méthode de mise en évidence de cryptosporidies dans les selles d'origine humaine et animale. Son rendement est comparé à celui de la plupart d'autres méthodes actuellement utilisées. Les méthodes de Köster et de Ziehl Neelsen modifiées montrent de meilleurs résultats. Toutefois, la coloration de Köster modifiée montre une meilleure définition de la structure interne des oocystes.

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