# STANDARDIZATION OF THE MODIFIED ZIEHL-NEELSEN TECHNIQUE TO STAIN OOCYSTS OF *CRYPTOSPORIDIUM* SP

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SUMMARY: Thirty calf faecal samples containing *Cryptosporidium* sp were used in the study. One g. of faeces was mixed with sufficient normal saline to obtain a smooth, even smear on a glass slide. A standard area was obtained by making the smear using a standard diameter platinum wire loop. Five smears of each feacal sample were made. The smears were stained by the modified Ziehl-Neelsen technique as described by HENRIKSEN & POLENS (1981). The influence of five different sulphuric acid concentrations (0.25%, 1%, 2%, 5% and 10% v/v), to differentiate the carbol-fuchsin staining, was tested on the number of oocysts counted in 25 microscopic fields of the smears, through a light microscope at magnification of 800 times. The greatest number of oocysts were detectable when the concentration of sulphuric acid was 2%. At this concentration the oocysts were easier to be found and identified. At lower concentrations (0.25% and 1%), the oocysts were deeply stained in red, the same colour as the background, and reduced the identification of the oocysts counted per area. At higher concentrations (5%, and 10%), the mordanting action of the acid destroyed many oocysts, and reduced the counts of properly stained ones. In conclusion, 2% sulphuric acid must be adopted to differentiate carbol-fuchsin staining in order to obtain an easier detection and the highest number of oocysts of *Cryptosporidium* sp counted in smears stained with the modified Ziehl-Neelsen technique.

KEY WORDS: *Cryptosporidium*, technique, standardization, oocysts, detection, Ziehl-Neelsen, differentiation.

## *INTRODUCTION*

*Cryptosporidium* is a minute coccidian parasite that characteristically inhabits the microvillous border of the host intestinal epithelial cells. Although it has been sporadically reported in the literature for the past 75 years, cryptosporidiosis has been recognized only recently as a potential cause of enterocolitis in animals and humans Among the farm livestock enteric cryptosporidiosis is found most commonly in the young ruminant (KIRKPATRICK & FARRELL, 1984; ANGUS, 1987) *Cryptosporidium* is a significant contributor to the calf diarrhoea complex. Epidemiological surveys carried out by ORTOLANI (1988), in the State of São Paulo, and GARCIA & LIMA (1994), in the State of Minas Gerais, Brazil, showed that 38% and 19% of all scouring calves, respectively, excreted large amounts of *Cryptosporidium* in the faeces.

The life cycle of *Cryptosporidium* species resembles that of other intestinal coccidians. The exogenous infective stage is the sporulated oocyst that contains four naked sporozoites. These sporozoites penetrate in the brush border of the intestine developing to trophozoites, schizonts, merozoites, macro and microgametocytes closing the life cycle, that can last three to 15 days, to oocysts that are passed in the faeces. The oocysts are round or elipsoidal and, in general, measure 4 to 5 μm in diameter but they have been noted to be as small as 2.5 to 3 μm or as large as 6 to 7 μm in diameter (BOUFASSA-OUZROUT *et alii* 1986, POHJOLA, 1986; KIRKPATRICK & FARRELL, 1984).

Many techniques were described to diagnose the cryptosporidiosis in man and animals. Most of them are related to the demonstration of the oocysts or any other form of the parasite in the faeces or histological samples. It is quite feasible to demonstrate serum antibody against *Cryptosporidium*, but it would be inappropriate to use serological methods in the face of an outbreak. This is caused by the inconvenience of a fortnight negative period, after the infection, when no antibody title is found (BOUFASSA-OUZROUT *et alii* 1986). Thus, any simple, rapid, inexpensive and reliable direct technique to identify oocysts in the faeces is recommended.

Some of the widely used techniques for detection of *Cryptosporidium* oocysts in the faeces include several forms of direct staining of the fecal smears and Sheather's sugar flotation-phase contrast microscopy. The former techniques have the advantage of requiring only an ordinary microscope, commonly found in any laboratory, while the Sheather's sugar

flotation technique requires more sophisticated equipment (KIRKPATRICK & FARRELL, 1984).

At first, it was recommended to stain the air-dried smears of liquified faeces, previously fixed with absolute methanol, with Giemsa (POHLENZ *et alii*, 1978). The oocysts stain blue and often contain red granules. Later on ANGUS *et alii* (1981), observed that either faecal yeasts and *Cryptosporidium* were stained similarly with Giemsa and both organisms were identical in size, and thus give rise to differential diagnostic problems. This technical limitation was overcome with the development by HENRICKSEN & POHLENS (1981) of a modified Ziehl-Neelsen technique which stains markedly *Cryptosporidium* oocysts, but yeasts. This technique has basically four steps: fixation of the smear with methanol; staining with concentrated carbol fuchsin (red color); differentiation with sulfuric acid and counterstaining with malaquite green. The oocysts appear as 3-6 μm large, densely stained red bodies clearly distinguishable against a green background. Although, this modified technique has been widely recommended, GARCIA *et alii* (1983), BOUFASSA-OUZROUT *et alii* (1986), POHJOLA (1986), DUBEY *et alii* (1990), some inconstant results concerning quantitative counting of oocysts were found. The most critical step of the technique is the differentiation of the smear with acid which may interfere with integrity of the stained partially acid-fast organisms (SONNENWIRTH *et alii*, 1980). The modified technique developed by HENRIKSEN & POHLENS (1981) suggested for the differentiation of the smears the use of sulphuric acid with a wide range of concentrations varying from 0.25% to 10%. Thus, additional studies information are necessary to know if different concentrations of sulfuric acid could interfere with the quantitative counting of oocysts in the smears.

This experiment was carried out to investigate the influence of the differentiation of faecal smears with five different concentrations of sulphuric acid on the counting of *Cryptosporidium* oocysts by the modified Ziehl-Neelsen technique as described by HENRIKSEN & POHLENS (1981).

## MATERIALS AND METHODS

Thirty calf faecal samples containing *Cryptosporidium* sp oocysts were used in this study. The samples were collected from different dairy farm as described by ORTOLANI (1988). One g of faeces was mixed with sufficient normal saline to obtain a smooth, even smear on a glass slide. A standard area was obtained by making the smear using a standard diameter platinum wire loop. Five similar smears were made from each single faecal sample. Smears were dried at 50°C, in a oven for one to two minutes, fixed first with 96% methanol, for three minutes, then by passing briefly through a flame. The staining was with concentrated carbol-fuchsin (0.34% fuchsin and 4% w/v phenol) for 20 minutes, without heating. To test for the optimum differentiation, the smears were washed, for 30 seconds, with one of the following sulphuric acid solution: 0.25%, 1%, 2%, 5% and 10% (v/v). Five per cent malaquite green was used to counterstain, for five minutes. At the end of each staining and differentiation the smears were rinsed with tap water. The stained smears were examined in a light microscope at magnification of 800 times, using the immersion objective. To compare the effects of the different solutions, the number of stained oocysts seen, randomly, in 25 microscopic fields were counted. The data was compared initially by analysis of variance with the application of Duncan's multiple range test.

## RESULTS

The number of oocysts counted in 25 microscopic fields in smears differentiated with different sulphuric acid concentration is shown in Table 1. The greatest numbers of oocysts were detectable when the concentration of sulphuric acid used to differentiate the carbol-fuchsin was 2%, followed by 1%, 5% and 10%. The lowest counting was detected in smears differentiated with 0.25% sulphuric acid ($p < 0.05$).

## DISCUSSION

The results clearly show that the differentiation in the acid-fast staining technique is a critical step in the efficiency of this methodology as stressed by SONNENWIRTH *et alii* (1980). The lowest counting of oocysts by the differentiation with 0.25% sulphuric acid solution was caused by the excessive impregnation of carbol-fuchsin in the background of the smear not removed adequately by the low concentration of the acid (Table 1). This excessive impregnation caused misdiagnosis of many oocysts deeply stained with the same colour as the background. On the other hand the smears differentiated with 5% and 10% sulphuric acid solution presented oocysts lightly stained in red and in most smears it was possible to detect fragments of the oocyst's membrane. The fragments were result of rupture of the organisms caused by the mordant action of the concentrated acid solution. The destruction of the oocysts by the acid certainly reduced the counts of properly stained ones. Usually, the Ziehl-Neelsen technique uses hydrochloric acid rather than sulphuric acid to differentiate the carbol-fuchsin staining (SONNENWIRTH *et alii*, 1980; ANGUS, 1987). Probably,

Table 1. Influence of the concentration of sulphuric acid used to differentiate the carbol-fuchsin staining on the mean number of *Cryptosporidium* oocysts counted in 25 microscope fields.

| SULPHURIC ACID CONC. (%) | MEAN Nº OOCYSTS DEVIATION | STANDARD |
|---|---|---|
| 0.25 | $10.6^{c}$ | 4.2 |
| 1 | $21^{b}$ | 5.4 |
| 2 | $26,7^{a}$ | 7.2 |
| 5 | $17.6^{b}$ | 6.9 |
| 10 | $17.3^{b}$ | 9.1 |

Letters with different subscripts are different ($p < 0.05$)

hydrochloric acid was chosen by the fact that is a slightly weaker acid than the latter one. Further studies are needed to compare the efficiency of both acids to differentiate the carbol-fuchsin staining.

The greatest number of oocysts were detectable when concentration of sulphuric acid used to differentiate the carbol-fuchsin was 2% (Table 1)

The use of this concentration was enough to remove the carbol-fuchsin from the background while the oocysts seen kept a bright and distinguishable red color against a green background. Practically, no fragments of membrane were seen in smears differentiated with this treatment.

This modified technique proved to be easy of being performed, rapid and highly practicable to detect oocysts of *Cryptosporidium* sp in faecal samples. Moreover, the technique allows the laboratory worker to investigate the presence of oocyst in several faecal samples concomitantly as well as the ready and clear identification of oocysts in the smears.

In conclusion, when a modified Ziehl-Neelsen technique is selected to stain faecal smears, for quantitative counting of *Cryptosporidium,* 2% sulphuric acid must be used, for differentiation of carbol-fuchsin staining.

## SUMÁRIO

Foram utilizadas neste trabalho trinta amostras fecais de bezerros excretando oocistos de *Cryptosporidium* sp. Um grama de fezes foi misturado com quantidade adequada de solução salina, a fim de ser obtida uma solução que permitisse a realização de esfregaços, em lâminas de vidro. Os esfregaços foram feitos em uma área padrão, utilizando-se para tal uma alça de platina. Para cada amostra fecal foram feitos cinco esfregaços idênticos. Os esfregaços foram corados segundo a técnica modificada de Ziehl-Neelsen, tal qual descrita por HENRIKSEN & POHLENS (1981). Foi estudada a influência de cinco diferentes concentrações de ácido sulfúrico (0,25%, 1%, 2%, 5% e 10% v/v) na diferenciação da coloração de carbol-fucsina, sobre o número de oocistos contado em 25 campos do esfregaço, através de leitura em microscópio óptico, em magnitude de 800 vezes. O maior número de oocisto foi constatado em esfregaços diferenciados com ácido sulfúrico a 2%. Nestes os oocistos apresentavam-se nítidos e de fácil reconhecimento. Menores concentrações deste ácido (0.25% e 1%) promoveram uma intensa coloração vermelha nos oocistos, os quais tingiram-se da mesma cor do fundo do esfregaço acarretando uma menor visualização e contagem de oocistos por área. Nas maiores concentrações (5% e 10%) a ação corrosiva do ácido destruiu muitos oocistos promovendo uma menor contagem destes. Pode-se concluir que deva ser adotada na diferenciação, da carbol-fucsina, solução de 2% de ácido sulfúrico, a qual permite a mais clara detecção de maior número de oocistos.de *Cryptosporidium* sp em esfragaços corados com a técnica modificada de Ziehl-Neelsen.

PALAVRAS-CHAVE: *Cryptosporidium*, técnica, padronização, oocistos, detecção, Ziehl-Neelsen, diferenciação.

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