Comparison Between The Efficacy Of Different Laboratory Methods For Diagnosis Of Cryptosporidium

Mohammed A. Kadir Nazakat F. Othman Yahya G. Salman
Kirkuk ,General Hospital, Kirkuk. Directory Of Health, Kirkuk
College Of Medicine, Tikrit.

Summary

This study was performed to detect the effective laboratory method for diagnosis of Cryptosporidium spp. in patients attending the laboratories of Al-Tameem General Hospital and Kirkuk Paediatric Hospital in Kirkuk city, for the period from the beginning of January to end of August 2000..

It was shown that the modified Ziehl-Neelsens stain (hot method) with methylene blue (counter stain) was most efficient in identification of Cryptosporidium oocysts (4.36%), followed by malachite green (counter stain) (1.94%) and modified Ziehl-Neelsen (cold method) with malachite green (counter stain), safranin-methylene blue, lugol's iodine preparation (mounting) (0.97%) for each and the modified Ziehl-Neelsen stain (cold method) with methylene blue (counter stain) was the least efficient (0.84%). .

مقارنة بين كفاءة الطرق المختبرية المختلفة في تشخيص طفيلي الابواغ الخبيئة

محمد عبد العزيز قادر نزاكت فخرالدين عثمان يحيى جرجيس سلمان
كلية الطب /تكريت مستشفى كركوك العام/كركوك دائرة صحة كركوك

الخلاصة

اجريت الدراسة للكشف عن طريقة مختبرية فعالة لتشخيص طفيلي الابواغ الخبيئة في المرضى المراجعين لمختبرات مستشفى التاميم العام ومستشفى الاطفال في مدينة كركوك للفترة من بداية كانون الثاني الى نهاية آب 2002 .تبين من النتائج ان طريقة زيل نلسن المحورة (الطريقة الحرارية) باستخدام صبغة الضد مثيلين الازرق ذات كفاءة عالية

في تشخيص اكياس البيض الخبيئة 4,36% ثم تلتها صبغة الضد ميلكايت الاخضر 1,94 %. اما بقية الطرق وهي طريقة زيل نلسن المحورة (الطريقة الباردة) باستخدام صبغة الضد ميلكايت الاخضر ، طريقة اللوكال أيودين وطريقة سفرانين مثيلين الازرق قد اظهرت كفاءة اقل بالنسبة الى الطريقة الاولى 0,97% اما بالنسبة الى طريقة زيل نلسن المحورة (الطريقة الباردة) مع صبغة مثيلين الازرق فقد كانت الاوطا 0,48%.

Introduction

Diagnosis of Cryptosporidium in faeces and sputum is done by direct smear technique with or without stains or by concentration methods (1). The parasite can be detected by staining air-dried, methanol-fixed faecal smears with Giemsa"s stain (2).

The first human case was diagnosed from intestinal biopsies. The stages of parasites seen are gametes or schizonts either immature or mature stage containing four to eight merozoites (2).

The most convenient method of laboratory diagnosis is direct recognition of oocysts in faeces. Giemsa and modified Ziehl-Neelsen stains were used originally but are inferior to safranin-methylene blue and phenol-auramin stains. The choice of method should be by personal evaluation (3& 4).

In the specimen stained with modified acid fast method, the oocyst stains red, whereas faecal yeast stains green when counter stained with methylene blue. The oocyst remains red but yeast stains blue (5). The effective staining method used for Cryptosporidium detection is the modified acid fast technique (Kinyoun, Ziehl-Neelsen) (1).

The acid fast trichrome stain yields results comparable to those obtained by Kinyoun and modified trichrome methods and considerably reduces the time necessary for microscopic examination (6). It has been shown that both acridine orange and bis-benzimide are more rapid, easier and less subjective than other methods (7).

There are two methods of concentration techniques, either using flotation method or sedimentation method, each one contains many types. The flotation method used with either saturated sucrose solution

or salt saturated with zinc sulfate. The sedimentation method used may be either with formal-ether solution or formaldehyde (8).

The present study aimed to detect the effective laboratory methods used in diagnosis of Cryptosporidium spp.

Materials And Methods

The study was conducted on 206 stool samples of patients attended the laboratories of Al-Tameem General Hospital and Kirkuk Paediatric Hospital in Kirkuk city, for period from first of January to end of August 2000. The stool samples were examined freshly and adequate amount of 2.5% potassium dichromate solution was added to specimen which examined later.

The staining used were lugol's iodine 1% (9), modified acid fast stain (hot method) (10), modified acid fast stain (cold method) (9) and safranine-methylene blue stain (10).

Statistical analysis was done to all data using Chi-square and t-test of homogenicity and independence to exclude source of variance between tested and control groups.

Results

Out of 206 stool samples examined using different laboratory methods, 26 were positive for Cryptosporidium spp. (12.62%).

Regarding the identification of Cryptosporidium infection using single, double and triple methods. Table (1), indicates that the rate of positivity was highest using single method (9.70%) followed by double methods (1.94%) and triple method (0.97%). Statistically differences between single, double and triple laboratory methods were significant ($P<0.05$).

Concerning the efficacy of different laboratory methods used for diagnosis of Cryptosporidium spp. Table (2) shows that the modified Ziehl-Neelsen stain (hot method) with methylene blue (counter stain)

was most efficient in identification of Cryptosporidium oocyst (4.36%) followed by malachite green counter stain (1.94%) and modified Ziehl-Neelsen cold method with malachite green counter stain, safranine-methylene blue and iodine preparation (mounting) (0.97%) for each and the modified Ziehl-Neelsen stain cold method with methylene blue counter stain was least efficient 0.48% ($P<0.05$).

Oocysts of Cryptosporidium were seen in distinct colour in each stain, the differentiation was done using light microscope on the power X100 oil immersion objective lens.

By using modofied Zeihl-Neelsen stain (hot method) counter stain methylene blue the oocyst appear as rounded shape, pale red surrounded with yeast (dark blue-violete) and bacteria (pale yellow). Three faint black dots were seen in the right anterior ends arranged like the letter capital (C) , it was fully sporulated (fig.1). While using malachite green, the oocyst appeared fully sporulated, spherical or ovoid shape reddish with a thin greenish outer layler , 2-3 vermiform sporozoites present in center of the oocyst , it was about ½ of the oocyst size . Other stool components were stained yellow colour with scattered greenish area containing yeast and bacteria (fig. 2).

In cold method, using methylene blue counter stain the oocysts were seen rounded, faint reddish, surrounded with dark blue background (fig.3) . While using malachite green as counter stain, (fig. 4), the oocyats appear as oval red, surrounded with a thin layer of faint yellow (zone), few dark granules were seen in the anterior portion of the oocysts, the yeast were stained with brownish colour surrounded with scattereds green back ground. Sporozoites were difficult to be disinguished in this method although the parasite was stained denser than in methylene blue counter stain in same method.

By using iodine mounting , the oocysts appear dark brownish, oval contain blak curved line with some thickness in both ends of the line. It is moon shaped occupied 1/5 of the oocysts size with some residual faint black dots were found. This may be parts of sporozoite, which was destroyed during slide preparation, faint yellowish layer that was

covering the parasite not clear visible. Monillia were seen as faint yellowish hallow with pale brownish background (fig. 5).

Using safranin-methylene blue phosphate stain, the oocysts were spherical-ovoid bright red, fully sporulated containing visible sporozoites, stained more ense in the central portion or region of the oocyst, surrounded with bacteria and yeasts on faint red background (fig.6).

Table 1. Comparison between single, double and triple methods used for detection of Cryptosporium sp.

Laboratory methods	No.+Ve	%	No.-Ve	%
Single method	20	9.70	186	90.30
Double method	4	1.94	202	98.05
Triple method	2	0.98	204	99.05
Total	26			

χ^2 =10.893 d. f = 2 ($p<0.05$)

Double laboratory methods:

Iodine + malachite green (hot method)=1

Malachite green (cold method) + malachite green (hot)= 1

Safranine + malachite green (cold) = 1

Safranine + malachite green (hot) = 1

Triple laboratory methods:

Methylene blue (hot)+malachite green (hot)+methylene blue (cold)=1

Table 2. Comparison between the efficacy of different laboratory methods for diagnosis of Cryptosporidium sp.

Laboratory methods	No. +Ve	%	No. -Ve	%
Modified Ziehl – Neelsen stain (hot) Methylene blue (counter stain)	9	4.36	197	95.63
Modified Ziehl – Neelsen stain (hot) Methylene green (counter stain)	4	1.94	202	98.05
Modified Ziehl – Neelsen stain (cold) Methylene blue (counter stain)	1	0.48	205	99.51
Modified Ziehl – Neelsen stain (cold) Methylene green (counter stain)	2	0.97	204	99.02
Safranine – methylene blue stain	2	0.97	204	99.02
Iodine preparation (mounting)	2	0.97	204	99.02
Total	20	9.70	186	90.30

χ^2 =6.30 d.f.= 5 (P<0.05)

Total number examined= 206 for each method
Total number positive = 26

Fig. (1) Cryptosporidium oocyst by using modified Ziehl-Neelsen (hot method) M.B. counter stain.

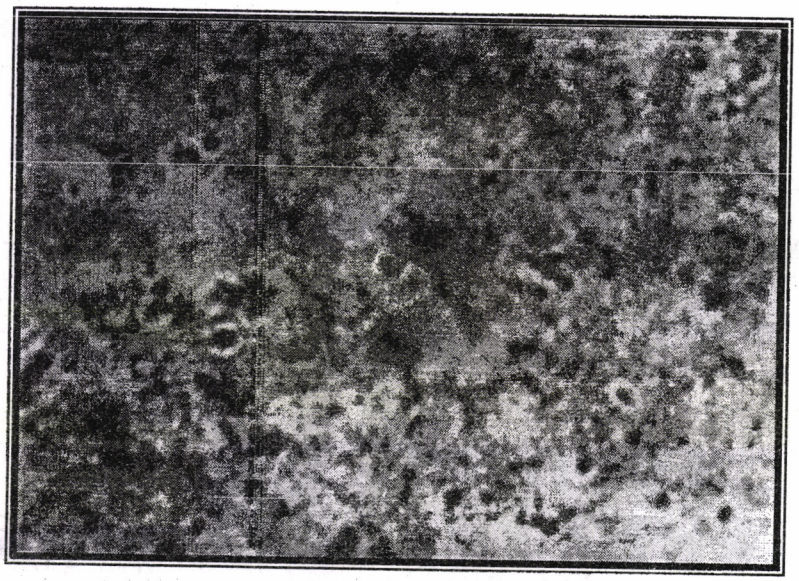


Fig. (2) Cryptosporidium oocyst by using modified Ziehl-Neelsen (hot method) M.G. counter stain.

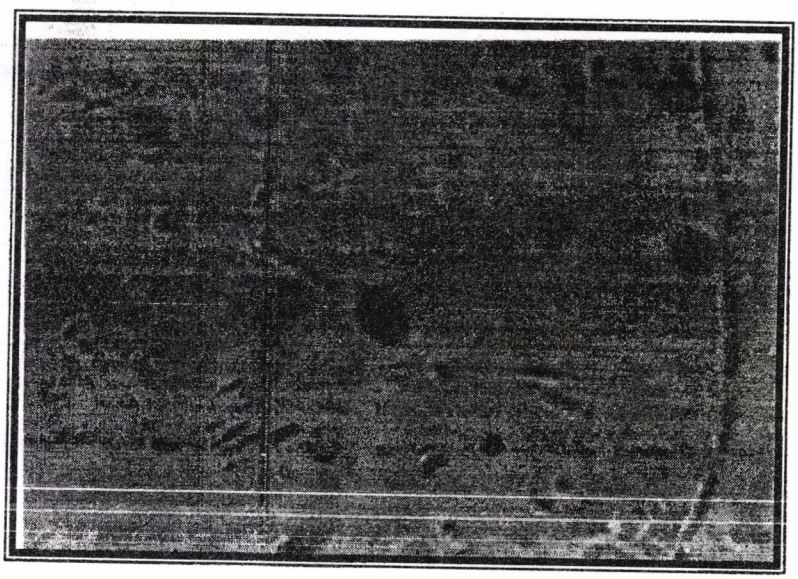


Fig. (3) Cryptosporidium oocyst by using modified Ziehl-Neelsen (cold method) M.B. counter stain.

Fig. (4) Cryptosporidium oocyst by using modified Ziehl-Neelsen (cold method) M.G. counter stain.

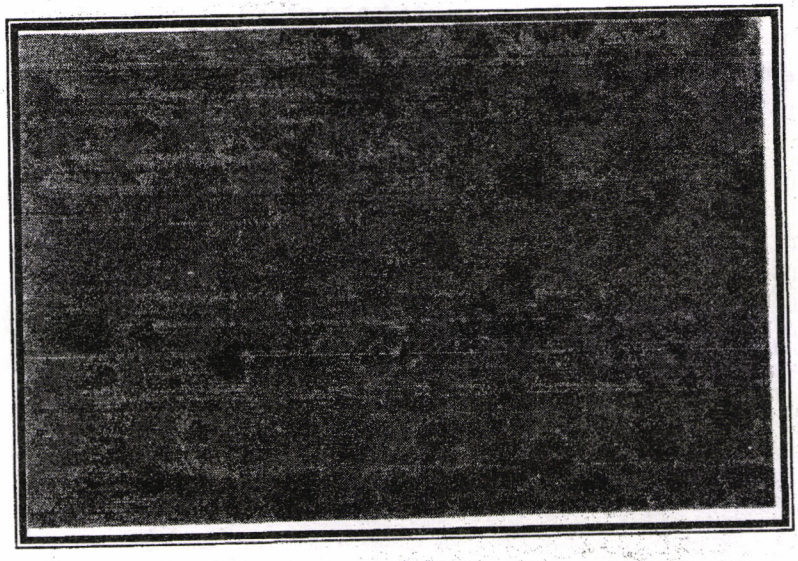
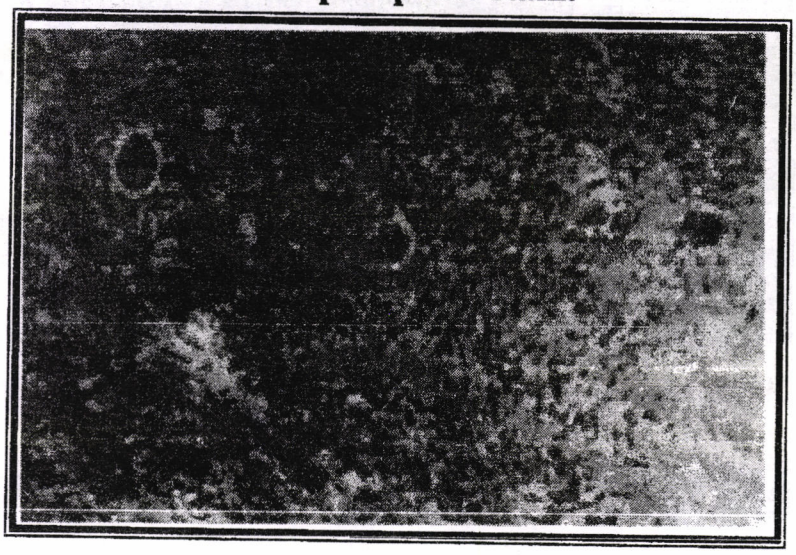


Fig. (5) Cryptosporidium oocyst by using lugol's iodine 5% (mounting)

Fig. (6) Cryptosporidium oocyst by using safranine methylene-blue phosphate stain.



Discussion

This study confirmed that the six methods used for diagnosis of Cryptosporidium spp. (Modified Ziehl-Neelsens hot method with counter stain methylene blue and malachite green, modified Ziehl-Neelsens cold method with counter stain methylene blue and malachite green, safranin methylene blue-phosphate, lugol"s iodone 5%) were useful for identification of the parasite.

The modified acid fast stain hot method was more efficient in diagnosis of Cryptosporidium spp. oocysts than others. This finding is in agreement with that reported by others (10,11). Ignatius et al. (12) mentioned that the acid fast stain represent the preferable method for the diagnosis of cryptosporidiosis.

Hammouda et al. (13) reported that the coccidian protozoa (Cryptosporidium oocyst) were clearly seen by using modified acid fast stain, but was not in agreement with that reported by Baxby et al. (14) and Youssef et al. (15) who showed that the modified acid fast method was less sensitive and slow method. The advantage of modified Ziehl-Neelsen:s (hot method) is that it has ability to differentiate Cryptosporidium from other pathogens, such as yeast and bacteria which take green colour rather than red colour when malachite green counter stain used (2). In addition to that it could recognize other protozoan parasites such as Giardia lamblia, Entamoeba histolytica and Blastocystis hominis and the slide can be kept for long period without destruction of the parasite (16).

In the present study the efficacy of Lugol:s iodine 5%, safranin-methylene blue and modified Ziehl-Neelsens cold method were the same rate (0.97%) but were less efficient than hot method.

Although lugol's iodine method is commonly used in all medical laboratories and requires short period for staining but its disadvantages are it require experience and good focusing of microscope, this may give mis diagnosis with yeast or monilia in the stool samples and the slides can not be kept for long period.

The low sensitivity of safranin-methylene blue stain than modified Ziehl-Neelsen"s hot method, may be due to type of fixative used, as acid alcohol was used as a fixative in safranin-methylene blue stain, while absolute alcohol was used as a fixative in Zeihl-Neelsen:s hot method. In addition to that 5% H2SO4 was used as decolorizing solution in modified Zeihl-Neelsen"s hot method, while in safranin method no decolorizing agent is required. This finding is not in agreement with that reported by Baxby (14) who found that the safranin-methylene blue is rapid and more sensitive than hot method.

The cold method was less sensitive than hot method, this might be due to heating was not performed in cold method, which lead to poor penetration of stain inside the parasite, or it might be due to long period of staining with carbol fuchsin stain (10 minutes) which decreased the sensitivity of cold method (9). In addition to that the decolorizing agent used in cold method was acid-alcohol (Hcl-methanol) which might be less efficient decolorizing agent than 5% H2SO4 which used in hot method.

From the results of this study it is concluded that the most efficient laboratory method for diagnosis of Cryptosporidium spp. was modified Ziehl-Neelsen"s (hot method)"

References

1. Casemore, D.P., Armstrong, M., Jakson, B., Nicholas, G. and Thom, B. (1984) Screening of Cryptosporidium in stool. Lancet: 31, 734.
2. Ma, P. and Soave, R. (1983) Three steps-.stool examination for cryptosporidiosis in ten homosexual men with protracted watery diarrhoea". J. Inf. Dis.: 147, 824-828.
3. Baxby D, Blundell, N and Hart, CA (1984) The development and performance of simple sensitive method for the detection of Cryptosporidium oocysts in faeces. J. Hyg.: 02, 317-23.

4. Payne, P., Lancaster, LA, Heinzman, M., and McCutchan, JA. (1983) Identification of Cryptosporidium in patient with the acquired immunodeficiency syndrome. N. Engl. J. Med.: 309, 613-14.
5. Markell, E.K., Voge, M. and John, D.T. (1986.) Medical Parasitology. 6 th. Edit. , Philadelphia, W.B. Saunders Co.: 331-337.
6. Ignatius, R; Lehmann, M; Miksits, K; Prgnath, T; Arvand, M Engelmann, E and Wagner, J. (1997) A new acid fast trichrome stain for simultaneous detection of Cryptosporidium parvum and microsporidial species in stool specimens. J. Clin.Microbiol:35, 446-9.
7. Brown, SM, McDonald, V, Denton, H. and Coombs, GH. (1996) The use of a new viability assay to determine the suceptibility of Cryptosporidium and Eimeria sporozoites to to respiratory inhibitors and extremes of pH. FEMS Microbiol. Lett: 142, 203-208.
8. Flec, S.L. and Moody, A.H. (1988). Diagnostic technique in medical parasitology. 1 st. edition Great .Britain, Butterworth Co.: 13.
9. World Health Organization. (1991) Basic laboratory method in medical parasitology. Geneva: 9-31.
10. Henrikson, S.A. and Pohlenz, J.F.L. (1981) Staining of Cryptosporidium by a Modified Ziehl-Neelsen technique. Acta Vet. Scand.: 22, 594-96.
11. Al-Yassin, S.T. (1999) A study of cryptosporidiosis in immunocomprimised patients. MSc thesis, College of Medicine, Tikrit Univ.
12. Ignatius, R., Elisenblatter, M., Regnath, T. (1997) Efficacy of different methods of low Cryptosporidium parvum oocysts numbers or antigen concentration in stool specimens. Eur. J. Clin. Microbiol. Infect. Dis.: 16, 732-6.

13. Haumouda, NA, Sadaka, HA, El-Gebaly, WM and El-Nassery, SM. (1996) Opportunistic intestinal protozoa in chronic diarrhoeic immunosuppressed patients. J. Egypt. Soc. Parasitol.: 26, 143-53.
14. Baxby, D., Hart, CA, Taylor, C. (1983) Human cryptosporidiosis a possible case of hospital cross infection. Br. Med. J., 278, 1760-1761.
15. Youssef, MY, Khalifa, AM and El-Azzonni, MZ (1998) Detection of Cryptosporidia in different water sources in Alexandria by monoclonal antibody test and modified Ziehl-Neelsen stain. J. Egypt. Soc. Parasitol.: 28, 487-96.
16. Manabe, YC, Clark, DP, Moore, RD. (1998) Cryptosporidium in patient with AIDS: correlates of disease and survival. Clin. Infect. Dis., Cited in Markell, John Krotosk, 1999.