Safranin staining of *Cyclospora cayetanensis* oocysts not requiring microwave heating

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Cyclospora cayetanensis is identified frequently as a cause of diarrhoea both in immunocompromised individuals and in those who are immunocompetent.[1-3] Although first detected as a human pathogen in 1979 by Ashford,[4] classification remained unclear for 16 years during which it has been reported as large *Cryptosporidium* spp., *Blastocystis hominis*, and a type of algae called 'cyanobacterium-like bodies'.[5-7] In 1993, when sporulation was demonstrated, it was identified as a coccidian and was given the name *Cyclospora* spp.[8]

Studies of the incidence of infection have been carried out worldwide;[9-11] however, the frequency of infection in many African nations is not known, since many laboratories do not have the facilities to use fluorescence microscopy or are unable to stain with microwave heating.

Staining methods used include Ziehl-Neelson, modified Kinyoun's acid-fast, and hot safranin. Acid-fast stains demonstrate *Cryptosporidium parvum* small oocysts (approximately 4-6 µm) and the larger (8-10 µm) *Cyclospora cayetanensis* oocysts; however, both acid-fast methods are variable in staining of parasites, leaving many unstained, and are less useful than the hot safranin stain used to detect *C. cayetanensis* oocysts.[12-14]

Fluorescence microscopy is used widely to detect 8-10 µm oocysts in wet mounts of concentrated faeces as the organisms show an autofluorescence[12] that is either green or blue depending on the excitation wavelength used. They can be differentiated from *Isospora belli*, both in fluorescent and acid-fast stained preparations, by their smaller size.

Since many laboratories in developing countries do not have fluorescence microscopes and appropriate filters, a simple method to find parasites is important. Here, a method is described to stain *C. cayetanensis* oocysts by heating in a water bath, which has been used to study faecal specimens from 80 patients with diarrhoea.

Faecal specimens were collected at Moi Teaching and Referral Hospital from patients presenting with diarrhoea. Information was collected on sex, age, food handling methods, water source and residence, together with clinical details on the frequency and severity of diarrhoea. Specimens were fixed in 10% formalin and polyvinyl alcohol preservative,[15] and those preserved in formalin were used to stain for *C. cayetanensis*.

The method of Visvesvara[14] was modified to provide staining equivalent to that achieved with the microwave technique. A specimen containing *C. cayetanensis* oocysts preserved in 10% formalin was used as a control. Various temperatures and staining times were used and water bath incubation was substituted for microwave heating. As heating at 60˚C on a slide warmer for up to 10 min had previously proved inadequate for staining,[14] a higher temperature was used. A water bath set to 80˚C was used to heat slides in safranin prepared as described previously.[14]

Incubation in safranin for 10 min failed to stain oocysts and therefore the time was increased to 15 min at 80˚C, preceded by 5 min at 85˚C. This procedure resulted in all oocysts in the smear being stained a bright red-orange, and no empty-looking oocysts were present. Initial trials at Indiana University of 10-min staining at 80˚C had produced good staining; however, longer incubation may have been needed at Eldoret (in Kenya) because of the higher altitude (2101 metres).

Of the 80 specimens evaluated, two contained *C. cayetanensis* oocysts. One was from a 29-year-old immunocompetent man who ate food from street kiosks, complained of abdominal pain but no previous episodes of diarrhoea. His specimen also contained *Endolimax nana* trophozoites. The other *C. cayetanensis*-positive case was a 40-year-old HIV-positive female who was both wasted and dehydrated, and described taking food from any source.

This study demonstrated that a water bath staining technique can be used to identify *C. cayetanensis* oocysts and, although only two positive cases were found in 80 patients studied, it will prove helpful in diagnosing this cause of diarrhoea. In reports of *C. cayetanensis* incidence in the USA, 'outbreaks' related to specific foods have been involved.[16-18] Delayed recognition of cryptosporidiosis in Milwaukee resulted from the lack of screening for *Cryptosporidium parvum* in many laboratories, and it is important to recognise this pathogen.[19]

The simple staining method described here may help to identify *Cyclospora cayetanensis* outbreaks related to contaminated foods in countries where laboratories do not have other means for oocysts identification. □

Development of the faecal staining study was part of a research project supported by a Fulbright Research Grant awarded to Professor Marilyn S. Bartlett, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, USA.

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Lectins as targeting agents – the *in vitro* binding of lectins to lesions in the eye and mouth

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Locating agents that bind to mucosal lesions of the eye and oral cavity would allow the selective delivery and retention of therapeutic or diagnostic agents. Lectins that bind specifically to the glycoconjugates present within a wound would provide an opportunity for such targeting.

Goldstein *et al*.[1] defined lectins as proteins or glycoproteins of non-immune origin that are capable of interacting with carbohydrates through at least two binding sites, agglutinating vegetable and/or animal cells, and precipitating polysaccharides, glycoproteins or glycolipids. The exact physiological role of lectins is unknown but they are implicated in many cell recognition and adhesion processes.[2]

Clear differences between cell-surface glycoconjugates on intact mucosal surfaces and diseased mucosa or cells from the underlying layers have been observed in previous studies. Differences in lectin binding were observed during the migration of the corneal epithelium,[3,4] in injured corneal endothelium during development,[5] in normal, scarred and lattice dystrophy corneas,[6] in keratoconus corneas,[7] and in normal, scarred and keratoconus corneas.[8]

Lectin binding has also been studied in oral squamous carcinoma,[9] in neoplastic and non-neoplastic oral white lesions,[10] in premalignant and malignant lesions of the oral cavity[11] and in regenerated junctional oral epithelium in the rat.[12]

A lectin that binds minimally to the intact mucosal surface might bind more avidly to the different glycoconjugates present in the underlying cells. Thus, the overall aim of this study is to investigate lectin binding to abrasions of the rat eye and oral mucosa, in order to identify a potential new method for targeting lesions with therapeutic and diagnostic agents. The current study uses an *in vitro* model of an abraded mucosal surface to investigate lectins identified from previous work[13-16] as binding both strongly and weakly to oral and ocular surfaces. Biotinylated lectins are employed and binding is detected using a streptavidin peroxidase/ diaminobenzidine staining procedure.

Biotinylated lectins from *Canavalia ensiformis*, *Erythrina corallodendron*, *Maackia amurensis*, *Lotus tetragonolobus*, *Narcissus pseudonarcissus*, *Solanum tuberosum* and *Sophora japonica* were purchased from Vector Laboratories (Peterborough, UK). The Diocleinae lectins (from *C. brasiliensis* and *Dioclea violacea*) were supplied as a gift by the University of Caera and biotinylated as described by Banchonglikitkul *et al*.[13] Sugar specificities for all lectins used in this study are shown in Table 1. Streptavidin peroxidase, 3′, 3′ diaminobenzidine tetrahydrochloride (DAB) and all other high-purity quality agents were purchased from the Sigma Chemical Company, Poole, UK. Lectin solutions were prepared as 10 mg/L in 0.05 mol/L Tris-buffered saline (TBS) containing 1 mmol/L $CaCl_2$ (pH 7.6).

Fresh tissues (eye balls, upper eyelids, buccal tissues and tongues) of Wistar rats, bred at the University of Portsmouth, were removed within two to three hours of death. All animals were reared in accordance with current UK government requirements.

All tissues were abraded by scratching with a blunt needle (1-mm diameter) across the central area of the tissue, in order to penetrate through the epithelium into the underlying connective tissue.

Table 1. Lectins used in the study

Source	Common name	Sugar specificity
C. brasiliensis	Brazilian bean	α-D-man, D-glc
C. ensiformis	Jack bean	α-D-man, D-glc
D. violacea	Brazilian bean	α-D-man, D-glc
E. corallodendron	Coral tree	β-D-gal-(1Æ4)-D-galNAc
L. tetragonolobus	Asparagus pea	α-L-fuc
M. amurensis	Maackia	Unknown
N. pseudonarcissus	Daffodil	Man
S. tuberosum	Potato	D-glcNAc oligomer
S. japonica	Japanese pagoda tree	β-D-galNAc, D-gal