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Crypto ID™

Detection of the Cryptosporidium Species and Strains by Polymerase Chain Reaction (PCR) Restriction Fragment Length Polymorphism (RFLP) Analysis

Submitter: Wastewater Facility ABC
Submitter #: 117
Source Molecular #: SM 0325
Sample Received: January 3rd, 2011
Date Reported: January 10th, 2011

SAMPLE

SM #	Client #	Cryptosporidium species / strains detected
SM 0325	117	Cryptosporidium parvum, genotype II (bovine genotype)**

**See Laboratory Comments below for explanation.

Laboratory Comments

DNA was extracted from the immunomagnetic separation (IMS) sample that was provided by the client. The DNA extract was analyzed with generic *Cryptosporidium* PCR primers and two different restriction enzymes to confirm the presence of *Cryptosporidium* and any specific species or strains.

Three results were obtained. First, generic *Cryptosporidium* was detected in the IMS sample. Second, the species *Cryptosporidium parvum* was identified using one of the restriction enzymes. Third, the strain labeled genotype II of *Cryptosporidium parvum* was confirmed with a different restriction enzyme. No other species or strains of *Cryptosporidium* were detected.

Cryptosporidium parvum, genotype II (bovine genotype) has been mainly associated with cattle. It is usually found in cattle farms harboring calves (young cattle), but not necessarily from adult cattle, which are probably more likely to harbor *Cryptosporidium andersoni*. *C. parvum* gentoype II has also been found on rarer occasions in other animals (such as sheep and goats) and in humans since it is pathogenic particularly for immunocompromised individuals. More information on *Cryptosporidium parvum* and the different genotypes can be found by consulting the references at the end of this report.[2,3,4,5,6,7]

In conclusion, it would seem that cattle are a source of the *Cryptosporidium* in the sample. Nonetheless, since some *Cryptosporidium* species or strains (such as *C. parvum* genotype II) are sometimes associated with other animal groups, it is important to strengthen the validity of the results with other microbial source tracking tests such as the Human Enterococcus ID™, Cow E. coli ID™, or Pig E. coli ID™ services. Furthermore other species or strains of *Cryptosporidium* might be present in the sample that were not detected with the restriction enzymes used. Additional restriction enzymes or other genomic techniques should be used to identify other species or strains of *Cryptosporidium* that might be present in the sample.

DNA Analytical Method Explanation

Immunomagnetic separation (IMS) of *Cryptosporidium* oocysts concentrate was either provided by the client or processed according to US EPA Method 1623.[1] DNA was extracted from a portion of the IMS concentrate by using a Chelex resin freeze-thaw method.[2] Briefly, IMS concentrates were centrifuged (10,000 X *g* for 3 min) and were resuspended in 50 µl of molecular-grade water. Water concentrates were mixed with 10 µl of 1:1 ratio (vol/vol) of Chelex resin–Tris-EDTA buffer and were subjected to eight cycles of freezing and thawing. DNA was recovered from the supernatant after a quick spin step.

Molecular characterization of *Cryptosporidium* species and genotypes was carried out by using a nested PCR-restriction fragment length polymorphism assay of the 18S small-subunit rRNA gene fragment and the restriction enzymes SspI and VspI. The PCR amplification reaction mixtures contained 1X PCR buffer (10X PCR buffer with 15 mM $MgCl_2$), 200µM (each) deoxynucleoside triphosphate, 100 nM (each) primer, 2.5 U of Hot Start Taq polymerase (Qiagen), and 5 and 50µl of DNA template in total 50- and 100-µl reaction mixtures, respectively. Forty PCR cycles (94°C for 45 s, 55°C for 60 s, and 72°C for 90 s) were carried out in an Eppendorf thermal cycler (Eppendorf AG) with an initial host start at 95°C for 15 min and a final extension at 72°C for 1 min 30 s. PCR products were analyzed on 1.5% agarose gels containing 0.5µg per ml of ethidium bromide in 1X Tris-borate-EDTA buffer (45 mM Tris-borate and 2 mM EDTA). Resulting bands were visualized by UV transillumination.

For the secondary PCR product, 5 µl of the primary PCR product was amplified with nested primers. Cycling conditions were identical to those used for the primary PCR. Secondary PCR products were purified by using the QIAquick PCR purification kit (Qiagen) and were eluted in Tris buffer (10 mM Tris-Cl, pH 8.5) prior to restriction fragment analysis to remove deoxynucleoside triphosphates, polymerases, salts, and primers.

For restriction fragment analysis, 20µl of the secondary PCR product was digested in a 25µl (total volume) reaction mixture containing 20 U of SspI for species diagnosis or 20 U of VspI for genotyping of C. parvum and the appropriate amount of restriction buffer at 37°C for 1 h. Digested products were fractioned on a 2.0% agarose gel and were visualized by ethidium bromide staining. The patterns of DNA bands were used to differentiate the species and genotypes of *Cryptosporidium* parasites according to methodology described by Xiao et al.[5,6]

DNA Analytical Theory Explanation

Cryptosporidium is a protozoa parasite affecting the gastrointestinal tract of humans and animals. They are shed in feces in the form of an oocyst. This protozoan can remain dormant for long periods in the oocyst form. It becomes active upon entering a host.

During this protective state (i.e. oocyst), *Cryptosporidium* is particularly difficult to remove from water systems. Ordinary water disinfection techniques cannot kill oocysts, and even the best filtration systems allow occasionally a few organisms to pass through.

Cryptosporidium causes a medical condition known as Cryptosporidiosis. Infections may be asymptomatic or may cause diarrhea, nausea, abdominal cramps, fever, vomiting and headaches. Immunocompetent individuals will usually recover from the illness within several weeks. However, immunocompromised individuals, such as HIV/AIDS or cancer patients, may be unable to remove the parasites from their systems and as a consequence, suffer debilitating illness and possible death.

Since the 80's, *Cryptosporidium* has become increasingly a major concern for water industry professionals. The problem became most visible when the Milwaukee, Wisconsin municipal water system suffered a severe *Cryptosporidium* outbreak in 1993. Over 400,000 persons fell ill and 70 individuals died as a result of this outbreak. Unfortunately, *Cryptosporidium* continue to wreck havoc both in large and small community water systems.

Recent advances in molecular biology techniques have allowed species and strains of *Cryptosporidium* to be identified.[2,3,4,5,6,7] To date, eight *Cryptosporidium* species have been regarded as valid on the basis of host specificity, pathogenesis, and oocyst morphology. These *include Cryptosporidium parvum in mammals, Cryptosporidium muris in rodents, Cryptosporidium* felis in cats , *Cryptosporidium wrairi* in guinea pigs, *Cryptosporidium baileyi* and *Cryptosporidium meleagridis* in birds, *Cryptosporidium serpentis* in reptiles, and *Cryptosporidium nasorum* in fish.[4,5,6]

Molecular typing tools have shown that two genotypes of *Cryptosporidium parvum* are responsible for Cryptosporidiosis. The human genotype (genotype 1) parasites have so far been found only in humans and primates, whereas the bovine genotype (genotype 2) parasites have been found in farm animals and some humans.[5,6]

Accuracy of the results is possible because the *Cryptosporidium* species and strain identification method uses PCR DNA technology. PCR allows quantities of DNA to be amplified into large number of small copies of DNA sequences. This is accomplished with small pieces of DNA called primers that are complementary and specific to the genomes to be detected.

Through a heating process called thermal cycling, the double stranded DNA is denatured and inserted with complementary primers to create exact copies of the DNA fragment desired. This process is repeated rapidly many times ensuring an exponential progression in the number of copied DNA. If the primers are successful in finding a site on the DNA fragment that is specific to the genome to be studied, then billions of copies of the DNA fragment will be available for detection by gel electrophoresis.

The gel electrophoresis apparatus uses an electrical field to distinguish different DNA fragments according to their molecular weights. Lighter DNA fragments will move farther along the gel than their heavier counterparts. At the end of the procedure different bands of accumulated DNA fragments will aggregate at different parts of the gel. It is this accumulation of DNA fragments that creates a band on the gel. Researchers use these bands to distinguish genomes of microorganisms such *Cryptosporidium*.

The PCR analysis can be further refined with the use of restriction enzymes. These enzymes when introduced into a DNA extraction will cut the DNA into smaller fragments. Restriction enzymes are a powerful genomic tool because they will always cut DNA at exact locations. As such, banding patterns will be heavier or lighter depending on where the restriction enzyme found its cut sites on the DNA molecule. Restriction enzymes exploit small variations in genomic sequences to create different banding patterns.

Consequently, these banding patterns provide a reliable indicator of the presence or absence of different species and strains of *Cryptosporidium*. It has been shown that these species or strains are associated predominately with certain animal groups such as cattle or humans. By identifying these varieties of different *Cryptosporidium*, it is possible to determine sources of *Cryptosporidium* pollution. Since some *Cryptosporidium* species or strains are sometimes found in other animal groups, it is important however to strengthen the validity of the results with other microbial source tracking tests such as the Human Enterococcus ID™, Cow E. coli ID™, or Pig E. coli ID™ services. Furthermore other species or strains of *Cryptosporidium* might be present in the sample that were not detected with the restriction enzymes used. Additional restriction enzymes or other genomic techniques should be used to identify other species or strains of *Cryptosporidium* that might be present in the sample.

References

[1] U.S. Environmental Protection Agency. 1999. **Method 1623: Cryptosporidium and Giardia in water by filtration/IMS/FA. EPA/821/R-99/006.** Office of Water, U.S. Environmental Protection Agency, Washington, D.C.

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