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Microbiology of the food chain — Detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits

Microbiologie de la chaîne alimentaire — Recherche et dénombrement de Cryptosporidium *et* Giardia *dans les légumes verts frais à feuilles et les fruits à baies*

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

Introduction

Cryptosporidium spp. and *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) are protozoan parasites that can cause enteric illness in humans. Both organisms are characterized by a robust transmission stage, the *Cryptosporidium* oocyst and the *Giardia* cyst, which can survive in moist environments for prolonged periods. These transmission stages are hereafter referred to collectively as (oo)cysts. *Cryptosporidium* oocysts in particular are highly resistant to chlorine at the concentrations used in the treatment of drinking water, and chemical disinfection of leafy green vegetables and berry fruits, where performed during processing, may also be ineffective. Consequently, the absence of vegetative bacteria on fresh produce as indicators of faecal contamination does not necessarily indicate the absence of (oo)cysts. No practical method exists to culture *Cryptosporidium* spp. and *Giardia duodenalis* for the purpose of detection, and therefore, in order to detect contamination with these parasites, direct removal of the (oo)cysts from the food sample must be performed, followed by visualization of the (oo)cysts by microscopy. The methods described in this International Standard are for determining whether *Cryptosporidium* and/or *Giardia* (oo)cysts are present on the surfaces of fresh produce and for their enumeration. This International Standard is based on published methods that have been tested in a multicentre collaborative trial. Alternative methods can be used following a demonstration of their equivalence with this International Standard following the protocol described in ISO 16140.[1]

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WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This International Standard specifies a method that is applicable for the detection and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts on or in food products that are described herein as fresh leafy green vegetables and berry fruits. With suitable controls, it may also be applicable for the examination of other fresh produce.

The microscopy descriptions are for *Cryptosporidium* spp. oocysts and *Giardia duodenalis* cysts of size ranges which include those species (*Cryptosporidium*) or assemblages (*Giardia*) known to be pathogenic to humans.

This method does not include any molecular analysis and therefore is not suitable for the determination of the species or genotypes/assemblages of *Cryptosporidium* oocysts and *Giardia* cysts. The method will detect all species and genotypes/assemblages that are known to be pathogenic for humans and also others that are not. For further identification, molecular typing assays are required. However, these cannot be reliably performed if process positive controls have been spiked into the samples, as the result of molecular typing assays will be obfuscated.

This method does not allow the determination of viability or infectivity of any *Cryptosporidium* oocysts and *Giardia* cysts which may be present.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 7218:2007, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
***Cryptosporidium* oocyst**
transmission stage of *Cryptosporidium* spp.

Note 1 to entry: Its detection is based on reaction with specific anti-*Cryptosporidium* antibodies and morphological characteristics as described in Clause 8.

3.2
***Giardia* cysts**
transmission stage of *Giardia* spp.

Note 1 to entry: Its detection is based on reaction with specific anti-*Giardia* antibodies and typical morphological characteristics as described in Clause 8.

3.3
fresh leafy green vegetable
plant leaves eaten as a vegetable, which have not been subjected to any process, except perhaps cutting and washing

3.4
fresh berry fruit
small, round or oblong, fleshy and juicy fruit, which has not been subjected to any process except perhaps cutting and washing

3.5
internal extraction control
(oo)cysts labelled with specific fluorogenic reporters that may be added in defined numbers to the sample prior to processing to assure that the method is operating properly

3.6
positive control
sample to which (oo)cysts have been added in defined numbers prior to extraction to verify that the method after the elution step is operating efficiently

3.7
negative control
sample having an equivalent quantity of material to the tested sample, which is considered to be free of (oo)cysts and processed in the same manner as the tested sample

4 Principle

The principle of the method is based on removal of the (oo)cysts from the sample by elution procedures, followed by concentration in the eluate by centrifugation and isolation by immunomagnetic separation (IMS). Detection of the (oo)cysts is performed by microscopy after labelling with specific monoclonal antibodies (mAbs) conjugated to a fluorochrome.

5 Reagents

5.1 Reagents required for eluting (oo)cysts from leafy green vegetables and berry fruits

5.1.1 Glycine buffer, pH 5,5 for leafy green vegetables (A.2.1).

5.1.2 Glycine buffer, pH 3,5 for berry fruits (A.2.2).

5.2 Reagents required for concentrating, fixing, staining, detection, and quality control

5.2.1 Methanol, analytical grade.

5.2.2 Paramagnetic beads, coupled with antibodies specific to the walls of *Cryptosporidium* oocysts and/or *Giardia* cysts.

5.2.3 Hydrochloric acid (HCl), 0,1 mol/l (A.3.1).

5.2.4 Sodium hydroxide (NaOH), 1 mol/l (A.3.2).

5.2.5 Fluorescently-labelled monoclonal antibodies (mAbs), against *Cryptosporidium* oocysts and/or *Giardia* cysts.

5.2.6 Immunofluorescence mounting medium (A.3.3).

5.2.7 4',6'-diamidino-2-phenylindole dihydrochloride dihydrate (DAPI), freeze-dried reagent.

5.2.8 DAPI stock solution (A.3.4).

5.2.9 DAPI working solution (A.3.5).

5.2.10 Phosphate buffered saline (PBS), pH 7,3 (A.1.1).

5.2.11 Non-fluorescing immersion oil.

5.2.12 Stock suspensions of *Cryptosporidium* oocysts and *Giardia* cysts (Annex B).

5.2.13 Suspensions of pre-labelled and enumerated non-viable *Cryptosporidium* oocysts and *Giardia* cysts.

The fluorochrome label shall be a different colour than that which is used for the detection of the target organism.

5.2.14 Parasite storage medium (A.3.6; A.3.7).

5.2.15 Demineralized and filtered water (ultrapure water type 1).

5.2.16 Fingernail varnish (for sealing coverslips onto slides as necessary).

6 Apparatus

This method requires common microbiological laboratory equipment (refer to ISO 7218) and, in particular, the following.

6.1 Tweezers, for handling fresh produce as necessary.

6.2 A paddle (peristaltic) blender and compatible filtered bags.

6.3 Swing out centrifuge, to accommodate at least 4 × 50 ml conical centrifuge tubes per run.

6.4 Glass Leighton tubes.

6.5 Rotating mixer, compatible with the Leighton tubes.

6.6 Magnetic clip stand, compatible with the Leighton tubes.

6.7 Magnetic clip stand, for microcentrifuge tubes.

6.8 Welled slides, with hydrophobic coating, wells capable of accommodating the 50 µl volume of processed sample after IMS, and coverslips of appropriate size.

6.9 Slide warmer tray, 37 °C to 42 °C incubator, or equivalent slide-drying apparatus.

6.10 Humidity chamber, lidded box containing damp absorbent material, e.g. a paper towel that can be held at the temperature and humidity appropriate for the immunofluorescent reagent.

6.11 Aspiration device, vacuum source equipped with liquid trap and pipette, or equivalent

6.12 Epifluorescence microscope with 20×, 40× objectives and 100× objective immersion lens and a calibrated eyepiece graticule (reticule), shall include fluorescein isothyocyanate (FITC) filter set (480 nm excitation, 520 nm emission filter) and DAPI filter set (375 nm excitation, >420 nm emission). If internal extraction controls are used, an additional filter set suitable for the fluorochrome will be required. Differential interference contrast (DIC) optics are advantageous. A photographic recording system attached to the microscope may be pertinent for recording positive or presumptive events.

6.13 FITC control slide, for evaluation of fluorescence intensity and verification of proper performance of the optical system of the fluorescence microscope.

7 Sampling and transport

7.1 Sampling

A procedure for sampling is not specified in this International Standard. See the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the relevant stakeholders (e.g. competent authorities, regulators, customers) come to an agreement on this subject.

7.2 Transport

Soft fruit samples and other delicate samples shall be handled carefully during transport to preserve their physical integrity (refer to ISO 7218:2007, 8.2).

7.3 Receipt of samples

Samples should be assessed for acceptance criteria using appropriate guidance as required within the purpose of testing (refer to ISO 7218:2007, 8.3). Fresh leafy green vegetables and berry fruits shall be regarded as perishable and analysis shall commence as soon as possible after acceptance. Criteria for rejection can include the presence of mould, decomposition of sample, or loss of sample integrity in the case of berry fruit.

7.4 Storage

Fresh leafy green vegetables and berry fruits should be stored refrigerated (between 4 °C and 8 °C), to reduce sample deterioration (refer to ISO 7218:2007, 8.4).

7.5 Preparation of test sample

The condition of the fresh produce shall be noted before the analytical procedure is commenced.

Test samples shall be at least 25 g.

It is recommended that no more than 100 g of any individual sample is analysed in a single procedure.

When analysing whole leafy green vegetables, such as lettuce heads, it is recommended that a random selection of intact clean leaves (exclude stems) from different parts of the plant should be examined.

For samples that are in small units, for example, raspberries, the sample shall consist of a random sample of these units.

8 Procedure

8.1 Removal of parasites from leafy green vegetables

a) Place sample in filtered bag. Avoid excessive handling. Use tweezers if necessary.

It is recommended that each sample is spiked with an internal extraction control suspension containing a defined number of pre-labelled and enumerated *Cryptosporidium* oocysts and/or *Giardia* cysts following the manufacturer's instructions. The control suspension should be pipetted on to the surface of the leaves; this should be done after the sample has been placed in the processing container. The fluorescent label used on the internal extraction control should differ from that used to detect the target (oo)cysts in the test samples. If internal extraction controls are not used, a positive process control sample is recommended (see 9.2).

b) Add 200 ml glycine buffer pH 5,5.

c) Process sample in paddle blender for 30 s at 200 rpm to 300 rpm.

d) Collect eluate into 50 ml conical-bottomed centrifuge tubes, dividing the eluate equally between them, ensuring that all the vegetable matter is retained in the filter. Squeeze the bag and the filter tightly in order to ensure that all the eluate is obtained from the sample. The eluate may be collected into a vessel before transferring to the centrifuge tubes.

Alternatively, the eluate can be transferred to a single 250 ml conical-bottomed centrifuge bottle, if an appropriate centrifuge/rotor is available for the subsequent step.

Rinsing the sample following washing could increase recovery efficiencies. After transferring the eluate from the filter bag, and while the tubes are being centrifuged [see point e)], add 10 ml 1 mol/l glycine buffer pH 5,5 to the sample and rinse by manipulating the sample from outside the bag. After removal of the supernatant from the centrifuged tubes, add the rinsate from the sample to the tubes. Add a further 10 ml 1 mol/l glycine buffer pH 5,5 to the bag, again manipulate produce and water from outside the bag, and add rinsate to tubes. Centrifuge as in point e).

e) Centrifuge the eluate at 2 500*g* maximum for 10 min with no braking.

NOTE 1 Centrifugation at 2 500*g* maximum can result in a very compact pellet. A lower speed of 1 100*g* maximum for 10 min with no braking has been reported to give at least equivalent recoveries of *Cryptosporidium* oocysts and *Giardia* cysts.

f) Remove the supernatant, by using a pipette and vacuum source, ensuring the pellet is not disturbed. If no pellet is visible, extra care shall be taken to ensure that parasites are not lost during aspiration. This can be done by leaving a small volume of liquid in the bottom of the tube.

g) Re-suspend the pellet in the residual liquid left in the bottom of each tube and combine the pellets into a single centrifuge tube.

h) Rinse the empty 50 ml conical centrifuge tubes with sterile distilled water and transfer the rinsate into the tube containing the combined pellets. Repeat this process until the volume of suspended pellet in the tube does not exceed the capacity of the tube.

i) Centrifuge the eluate at 2 500*g* maximum for 10 min with no braking.

NOTE 2 Centrifugation at 2 500*g* maximum can result in a very compact pellet. A lower speed of 1 100*g* maximum for 10 min with no braking has been reported to give at least equivalent recoveries of *Cryptosporidium* oocysts and *Giardia* cysts.