chloride enhancement of DAB for use in histochemical studies of chorioretinal specimens or other tissues where melanin might be an obstructive factor. □

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Molecular detection and identification of *Cryptosporidium* species in lettuce employing nested small-subunit rRNA PCR and direct automated sequencing

M. SHIGEMATSU[*,†], Y. NAGANO[*], B. C. MILLAR[*], F. KENNY[‡], C. J. LOWERY[§], L. XIAO[¥], J. R. RAO[#], V. NICHOLSON[‡], M. WATABE[*], N. HEANEY[*] O. SUNNOTEL[§], K. McCORRY[*], P. J. ROONEY[*], W. SNELLING[§], J. S. G. DOOLEY[§], J. S. ELBORN[§], M. MATSUDA[†] and J. E. MOORE[*]

[*]Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast, Northern Ireland; [†]Laboratory of Molecular Biology, School of Environmental Health Sciences, Azabu University, Sagamihara, Japan; [‡]Sligo Public Health Laboratory, Sligo, Ireland; §School of Biomedical Science, University of Ulster, Coleraine, County Londonderry, Northern Ireland; [¥]Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA; [#]Applied Plant Science Research Division, Agri-Food and Biosciences Institute, Newforge Lane, Belfast; and [§]Department of Respiratory Medicine, Queen's University, Level 8, Belfast City Hospital, Lisburn Road, Belfast, Northern Ireland

Human cryptosporidiosis emerged as an important gastrointestinal infection during the 1990s, due to the ingestion of mainly contaminated water and to a lesser extent foodstuffs containing the protozoan parasite *Cryptosporidium parvum*. A limited number of studies have examined the occurrence of the parasite on vegetables, including lettuce.

Fresh lettuces (n=50; iceberg and round) were obtained at retail sale in Northern Ireland during 2005 and were examined for the molecular presence of *Cryptosporidium* spp. by nested polymerase chain reaction (PCR) amplification of the small ribosomal RNA (rRNA) subunit. Just one lettuce was positive for this parasite, which was further confirmed by direct automated sequencing of the small rRNA subunit amplicon as either *C. parvum* or *C. hominis*, with 361/361 (100%) bases identified to either of these species.

Horticultural producers of lettuce should therefore place special emphasis on developing suitable and efficient Hazard Analysis and Critical Control Points (HACCP) strategies for the critical control of cryptosporidial oocysts, depending on the type of unit operation employed and the lettuce being processed.

Correspondence to: Dr. John E. Moore
Email: jemoore@niphl.dnet.co.uk

Fertilisation of horticultural crops, including lettuce, with manure from cattle and sheep containing viable oocysts of *Cryptosporidium* represents a significant public health risk to the contamination of these produce. Recently, Hutchinson *et al.*[1] demonstrated that persistence of *C. parvum* oocysts in farmyard wastes on a grass pasture ranged from eight to 31 days for a 1-log reduction in *C. parvum* levels and demonstrated that the protozoans were significantly more persistent than the bacteria. Oocyst recovery was more efficient from wastes with a lower dry matter content.

These workers concluded that horticultural crops might become contaminated with this parasite. Consequently, with a growing consumer trend away from intensively produced crops towards organic produce, this could increase the likelihood of contamination and the threat to public health.

In addition, horticultural produce may become contaminated by free-living wildlife, including wild birds such as gulls[2], as well as insects including flies[3] and dung beetles,[4] which have been shown previously to be carriers for the parasite, Furthermore, in certain countries, particularly the Far East, the practice of adding untreated human sewage (night soil) to fertilise horticultural crops is of considerable risk for the spread of *Cryptosporidium* to humans who ingest such produce.[5]

The main cause for concern in the horticultural industry is the risk of irrigation with untreated water of crops that do not require any thermal processing prior to consumption. As untreated surface waters are frequently contaminated, use of such supplies should not be used without adoption of control mechanisms to eliminate viable oocyts.

To date, there have been no reports on the contamination of lettuces consumed on the island of Ireland. Additionally, there have been limited reports on the use of molecular techniques, such as 18S rRNA PCR and sequencing, for the detection of this parasite from food produce. Consequently, the aim of this study is to employ molecular techniques to determine the occurrence and identification of the parasite on lettuces on retail sale in Ireland.

Fresh lettuces (n=50; Class I) were purchased from retail supermarkets in Northern Ireland during August to December 2005. These consisted of produce from Northern Ireland (iceberg lettuce [n= 8], round lettuce [n=12]) and from Spain (iceberg lettuce [n=30]). Produce type and country of origin were noted for all produce examined. Lettuces were transported to the laboratory for examination and were examined within four hours of purchase.

Lettuces were sliced with a sterile blade and a portion (25 g) was placed in a stomacher bag, to which 0.1% (w/v) peptone saline (139.5 mL; Oxoid CM0733, Basingstoke, UK) incorporating sodium laurylsulphate (0.22 g/1000 mL; Sigma, St. Louis, MI, USA) was added and pulsified (Microgen Bioproducts, Camberley, Surrey, UK) for 15 sec. After pulsifiying, the suspension was further agitated by stomaching for 120 sec (Stomacher 400 Circulator, Seward, Thetford, Norfolk, UK) on a high-speed setting in filter-lined stomacher bags (Seward).

The filtrate was decanted into sterile centrifuge tubes (50 mL, Falcon, Becton Dickinson, Oxford, UK) and centrifuged at 4000 x*g* for 10 min at room temperature (Labofuge 400,

Heraeus, Germany), after which the supernatant was decanted and 1xTris–HCl (200 µL, pH 8.0) was added. This was vortex-mixed to remove any pelleted oocysts. The supernatant was transferred to a clean 1.5 mL centrifuge tube and the genomic DNA extracted. Positive and negative extraction controls, containing purified oocysts (*C. parvum*) and sterile distilled water, respectively, were set up for each batch of extractions. *C. parvum* (reference strain) was obtained from Moredun Scientific, Penicuik, Scotland, UK.

Genomic DNA was extracted from oocysts by six cycles of freeze-thawing (liquid N_2 [1min] + 100°C [1min]), followed by extraction using the Roche High Purity PCR Template Kit (Roche Diagnostics, UK), following the manufacturer's instructions. Extracted DNA was stored at –80°C until required.

All molecular procedures were carried out in accordance with the good molecular diagnostic protocol detailed in the guidelines of Millar *et al*.[6] Nested PCR amplification of the small subunit 18S rRNA gene was performed, targeting a polymorphic region of the 18S rRNA gene of *Cryptosporidium* species. Briefly, using PCR conditions previously described by Xiao *et al*.[7] for primary amplification, reaction primer pair A and B (Table 1) were used to obtain amplicons of approximately 1325 bp. The internal primers C and D were then used to amplify a 820-bp region of the primary PCR product (Table 1). During each run, molecular grade water was included randomly as negative controls, and appropriate DNA template from *C. parvum* was included as a positive control.

Following amplification, 15-µL samples were removed from each reaction mixture and examined by electrophoresis (80 V, 45 min) in gels composed of 2% (w/v) agarose (Gibco Life Technologies, Paisley, UK) in TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]), stained with ethidium bromide (5 µg/100 mL). Gels were visualised under ultraviolet (UV) illumination using a gel image analysis system (UVP Products, Cambridge, England), and all images were archived as digital (*.bmp) graphic files.

Amplicons (25 µL) for sequencing were purified with a QIAquick PCR purification kit (Qiagen, UK) and eluted in Tris–HCl (10 mmol/L, pH 8.5) prior to sequencing. The Cy-5'-labelled primer C was prepared and used for sequencing in the forward direction. Amplicons were sequenced on the ALF Express II (Amersham-Pharmacia, Buckinghamshire, England) employing the Thermo Sequenase fluorescence-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, UK; RPN 2438) at 96°C for 1 min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec, 60°C for 5 sec, followed by a 4°C hold.

The resulting sequences from both reactions were aligned and a sequence of approximately 1350 bases was obtained. This sequence was compared with those stored in GenBank using the BLASTn alignment software (www.blast.genome.ad.jp/). Sequence homology identity was determined in accordance with the criteria described previously.[8]

One lettuce (Class I iceberg lettuce) was positive by nested small subunit RNA PCR for the presence of *Cryptosporidium* sp. The sensitivity of the PCR assay was 11 oocysts. The identification was confirmed by direct automated sequencing of the nested rRNA PCR amplicon (a 361 bp fragment), which is deposited in GenBank as *Cryptosporidium* sp. FSPB Lettuce 41 (accession number DQ530351). BLASTn analysis identified the sequence as 361/361 bases (100%) for either *C. parvum* (DQ054817) or *C. hominis* (AJ849464).

Most previous studies that have examined the occurrence of this parasite on lettuces and other vegetables employed immunomagnetic separation (IMS), followed by serological and microscopical detection of oocysts by fluorescein isothiocyanate (FITC) staining.[9–12] These studies report contamination rates of 2.5–14%, as detailed in Table 2. Likewise, the present study has demonstrated a contamination rate similar to previously published data.

The main cause for concern in the horticultural industry is the risk posed by irrigation of crops that do not require thermal processing prior to human consumption. As untreated surface waters may be contaminated, use of such supplies should not be adopted without appropriate control mechanisms to eliminate viable oocyts. Chlorination alone has not been successful in eliminating waterborne *Cryptosporidium* oocysts, as they are resistant to the biocidal activity of free chlorine. Furthermore, intake of mains water from the public supply should be monitored carefully, as there have been several reports of such supplies being positive for the organism.[13]

Consequently, use of contaminated water irrigation, as either an ingredient or in cleaning/rinse systems, without any controls for the elimination of oocyst, may lead to the production of contaminated foodstuffs. Armon *et al.*,[14] in a comparison of surface and subsurface irrigation methods, demonstrated the presence of *Cryptosporidium* oocysts (0–640 oocysts/g) in soil at different depths (0–90 cm) and found the highest prevalence (80–10,000 oocysts/0.5 kg) on zucchini, which has a sticky and hairy outer surface. Furthermore, in a study in Central America, Thurston-Enriquez *et al*.[15] showed that 36% of irrigation waters tested positive for *Cryptosporidium* sp., with 227 oocysts/ 100 L.

Table 1. Oligonucleotide primers employed in this study.

Primer	5'-3' nucleotide sequence	Comments	Gene copy number	Reference
A	TTC TAG AGC TAA TAC ATG CG	Forward primer used in primary 18S rRNA nested PCR	5	7
B	CCC TAA TCC TTC GAA ACA GGA	Reverse primer used in primary 18S rRNA nested PCR	5	7
C	GGA AGG GTT GTA TTT ATT AGA TAA AG	Forward primer used in secondary 18S rRNA nested PCR and sequencing	5	7
D	AAG GAG TAA GGA ACA ACC TCC A	Reverse primer used in secondary 18S rRNA nested PCR and sequencing	5	7

Table 2. Previous reports of the occurrence of *Cryptosporidium* spp. on vegetables.

Country	Comments	References
Costa Rica	Cilantro leaves 5.2% (4/8), Cilantro roots 8.7% (7/80), lettuce 2.5% radish (1.2%), carrot (1.2%), tomato (1.2%), cucumber (1.2%)	9, 10
Peru	14.5% of vegetables examined contained *C. parvum* oocysts	11
Norway	19/475 (4%) fruits and vegetables examined were positive (five lettuces, 14 mung bean sprouts) Low oocyst density (three oocysts/100 g food)	12

In conclusion, this study has demonstrated a low (2%) occurrence of *Cryptosporidium* sp. in the lettuces examined. As this is the first report of its occurrence on lettuces from the island of Ireland, further and larger studies are now required to examine the contamination of local and imported lettuce for the presence of this organism. However, horticultural producers of lettuce should evaluate their operations in terms of HACCP to prevent the contamination of their crop with this parasite of public health significance. □

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