Intestinal protozoan parasites in Northern India – investigations on transmission routes

Philosophiae Doctor (PhD) Thesis

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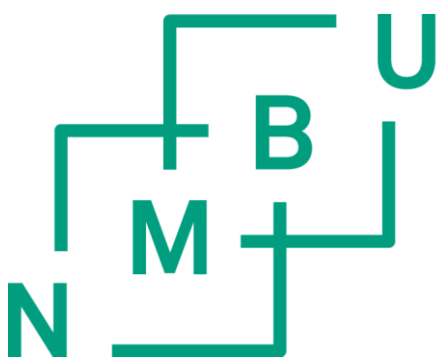

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To Jenny, Vilmer, Viljar and Ivo.

"India can do it. People of India can do it."

– PM Modi on Swachh Bharat Abhiyan

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Mera bhedh, I`ll always hate you the least.

Abbreviations

18S rDNA: 18S ribosomal DNA

AR: Attributable Risk. the portion of disease incidence in the exposed that is due to the exposure.

cowp: Cryptosporidium oocyst wall protein.

CPG: Number of *Giardia* cysts per gramme feces.

DAPI: 4´, 6-diamino-2-phenylindole.

DNA: Deoxyribonucleic acid.

DAPI: 4′6 diamidino-2-phenylindole, a non-specific fluorescent stain that binds to double-stranded DNA.

ELISA: Enzyme-linked immunosorbent assay.

gdh**:** Glutamate dehydrogenase.

GP60: 60kDa glycoprotein.

IFAT: Indirect fluorescent antibody test.

Immuno-magnetic separation (IMS): the separation of oocysts and cyst by para-magnetic beads covered with specific antibodies.

ISO: International Organization for Standardization, a worldwide federation of national standards bodies.

Monoclonal Antibodies (mAbs): monospecific antibodies that are made by identical immune cells that are all clones of a unique parent cell

mRNA: Messenger RNA

OPG: Number of *Cryptosporidium* oocysts per gramme feces

Polymerase Chain Reaction (PCR): Method used to amplify, and therefore enables detection and sequencing of specific strands of nucleic acids (DNA or RNA)

rRNA: the RNA component of the ribosome

SSU rRNA: Small sub-unit ribosomal RNA

Tpi: Triosephosphate isomerase

List of research papers

Paper I

A reduced cost-approach for analyzing fresh produce for contamination with *Cryptosporidium* oocysts and/or *Giardia* cysts.

Authors: Kjersti Selstad Utaaker, Qirong Huang and Lucy J. Robertson.

Published: *Food Research International* (2015) **77** 326-332.

Paper II

Keeping it cool: Survival of *Giardia* cysts and *Cryptosporidium* oocysts on lettuce leaves

Authors: Kjersti Selstad Utaaker, Eystein Skjerve and Lucy J Robertson.

Published: *International Journal of Food Microbiology* (2017) **255** 51-57.

Paper III

Checking the detail in retail: Occurrence of *Cryptosporidium* and *Giardia* on vegetables sold across different counters in Chandigarh, India.

Authors: Kjersti Selstad Utaaker, Himanshu Joshi, Anil Kumar, Suman Chaudhary and Lucy J. Robertson

Published: *International Journal of Food Microbiology* (2017) **263** 1-8.

Paper IV

Goats in the city: prevalence of *Giardia* and *Cryptosporidium* in extensively reared goats in northern India

Authors: Kjersti Selstad Utaaker, Nina Myhr, Rajinder S. Bajwa, Himanshu Joshi, Anil Kumar and Lucy J. Robertson.

Submitted: *Acta Veterinaria Scandinavica*.

Paper V

Is drinking water making waves in Chandigarh? Occurrence of *Cryptosporidium* and *Giardia* in potable water sources.

Authors: Kjersti Selstad Utaaker, Himanshu Joshi, Anil Kumar, Lucy J. Robertson.

Submitted: *Journal of Water and Health*

Paper VI

Prevalence and zoonotic potential of intestinal protozoans in bovines in Northern India

Kjersti Selstad Utaaker, Suman Chaudhary, Rajinder S. Bajwa, Lucy J. Robertson.

Submitted: *Veterinary Parasitology – Regional Studies and Reports*.

Paper VII

Not just a walk in the park: prevalence and seasonal variation of parasites in faeces shed in recreational parks in Chandigarh, India.

Authors: Kjersti Selstad Utaaker, Kristoffer Relling Tysnes, Marie Myklatun Krosness, Lucy J. Robertson.

Manuscript.

List of additional papers

Paper I

Climate change and foodborne transmission of parasites: a consideration of possible interactions and impacts for selected parasites.

Authors: Kjersti Selstad Utaaker and Lucy J. Robertson.

Published: *Food Research International* (2015) **68** 16-23.

Paper II

Keeping parasitology under the One Health umbrella.

Authors: Lucy J. Robertson, Kjersti Selstad Utaaker, Kapil Goyal, Rakesh Sehgal

Published: *Trends in Parasitology* (2014) **30.8** 369-372.

Summary

Cryptosporidium and *Giardia* are protozoan parasites that have been confirmed as major causes of diarrhoea, particularly in children. They represent a significant, but often neglected, threat to public health, and particularly so in developing countries. They are able to cause widespread human and animal disease, and both protozoa contain species that are able to infect a wide range of host species, and are well-suited to cross the human ↔ animal boundaries. The robust transmission stages of both parasites, along with their high excretion rates and low infective dose, means that they can be transmitted through contamination of drinking water and fresh produce, as well as directly.

Despite these facts, there are fewer reports on occurrences and outbreaks of cryptosporidiosis and giardiasis in developing countries, where there is no surveillance of contamination of the water supply, the fresh produce chain is not properly monitored, and animals roam with less restriction than in developed countries making the human ↔ animal boundaries fade. The reasons for this are many, and probably include diagnostic difficulties, lack of reporting, and an absence of investigation; it is unlikely to reflect that these infections occur more frequently in developed countries.

This thesis consists of an experimental part and a survey part. The experimental part has a focus on affordable health, where expensive standard methods were modified and made accessible as cheaper options for analysis of fresh produce and drinking water for contamination with *Cryptosporidium* and *Giardia*. Also, the survival of infective stages of *Giardia* and *Cryptosporidium* on experimentally contaminated fresh produce was assessed; *Giardia* cysts were less capable of survival when stored at room temperature than refrigerated, whereas *Cryptosporidium* oocysts survived well both when refrigerated and at room temperature. This may partly explain the few documented foodborne outbreaks of giardiasis.

I also present five survey studies that investigate the epidemiology, as well as occurrence and prevalence, of these protozoans in Chandigarh, a city in Northern India. Chandigarh has a structured outlay and a relatively well-developed infrastructure, although the city is facing problems that can be found in many situations in the developing world, such as

overpopulation, slum areas, poor water quality and access, sanitation difficulties from handling of sewage to the level of toilet availability, roaming stray animals, and enormous cultural and socio-economic divisions among both human and animals.

Traditional markets and street vendors, as well as modern supermarkets, sell fresh produce in all areas of Chandigarh, and it seems that traditional retail outlets have the lowest occurrence of produce contaminated with parasites in comparison to the modern ones. This may represent a reflection of a developing country mimicking the developed in terms of trade, but the infrastructure perhaps not yet being ready to handle these changes.

Like many Indian cities, water shortage is a common problem, even in Chandigarh. Contaminated water seems to affect the population living in higher density areas, although low levels of parasites were found in most positive samples. Notably, one sample from the slum area, where the residents commonly receive their drinking water in transported tanks, contained a high number of *Giardia* cysts.

In backyard goats, *Giardia* was a common parasite, and the isolates found were the same as those commonly found in humans, and differing from those usually identified in goats living in the developed world. This result suggests that a “human ↔ goat”, rather than the western “goat ↔ goat” transmission cycle may occur more frequently in this situation, underlining the lack of basic sanitary facilities in these human and goat populations, which reduces the barrier for infection between species.

However, the situation was completely different in dogs roaming the recreational parks in Chandigarh, where the majority of *Giardia* isolates were canid-specific. Dogs are not traditionally approached as pets in Indian culture, and stray dogs, especially, are avoided. This may be due to a fear of being bitten and the likelihood of rabies. Thus, even though human and dogs roam the same parks in Chandigarh, they do not share the same intestinal protozoans.

Cattle in India are both worshipped and neglected, and some bovines roam the streets alongside their human counterparts scavenging for food. Interestingly, these holy creatures did not harbour many *Giardia* isolates with zoonotic potential, but *Cryptosporidium* subtypes previously found in humans in Chandigarh was also found in calves.

Taken together, these studies provide information on possible transmission pathways of *Cryptosporidium* and *Giardia*. It seems that cultural and socioeconomic levels also play a part on transmission routes, and that although waterborne and foodborne outbreaks of cryptosporidiosis and giardiasis are rarely reported or published from developing countries, the potential is certainly there, and outbreaks may be grossly underestimated and underreported.

Sammendrag (Norwegian summary)

Giardia og *Cryptosporidium* er parasittiske protozoer som har etablert seg som en av hovedårsakene til diarè hos mennesker, og da spesielt hos barn i utviklingsland. Der representerer de en signifikant, men ofte neglisjert, trussel for folkehelsen. De kan også forårsake utbredt sykdom hos dyr, og er velegnet til å krysse smittebarrierer mellom arter. På grunn av deres robuste overføringsstadier, høye ekskresjonsrate og lave infeksjonsdose, er disse parasittene svært effektive smittespredere og de kan overføres via kontaminerte drikkevannskilder og ferske råvarer, i tillegg til direkte smitte.

Til tross for av at dette har vært lenge kjent, så er det færre rapporter om forekomster og utbrudd av kryptosporidiose og giardiose i utviklingsland, hvor hverken vannforsyning eller ferskvarekjeden overvåkes i samme grad som i utviklede land. I tillegg kan ofte dyr streife med mindre begrensninger enn i utviklede land, noe som resulterer i at smittebarrierene mellom mennesker og dyr blir mindre robuste. Årsakene til dette er mange og sammensatte, og sannsynligvis inkluderer de mangel på ressurser og utstyr til å utføre diagnostikk, manglende rapportering og mangel på overvåkning.

I denne doktorgraden presenterer jeg en eksperimentell del og en deskriptiv del. Den eksperimentelle delen fokuserer på utvikling av rimeligere diagnostiske metoder, der kostbare standardiserte metoder ble modifisert og gjort tilgjengelige som billigere alternativer for analyse av ferske råvarer og drikkevann for påvisning av kontaminering med *Cryptosporidium* og *Giardia*. Overlevelsen av infektive stadier av *Giardia* og *Cryptosporidium* på eksperimentelt kontaminerte ferske råvarer ble også evaluert; *Giardia*-cyster hadde lavere viabilitet når de ble lagret ved romtemperatur enn kjølt, mens *Cryptosporidium*-oocystene overlevde både når de var kjølt og ved romtemperatur. Dette kan delvis forklare de få dokumenterte matbårne utbruddene av giardiose.

Denne tesen består i tillegg av fem deskriptive studier som undersøker epidemiologi, forekomst og utbredelse av disse protozoene i Chandigarh, en by i Nord-India. Chandigarh har en strukturert arkitektur og en relativt velutviklet infrastruktur, selv om byen står overfor problemer som er vanlige i utviklingsland; som overbefolkning, slumområder, dårlig vannkvalitet og tilgang på drikkevann, sanitetsproblemer ved håndtering av avløpsvann og

tilgjengelighet på toaletter, eierløse dyr og enorme kulturelle og sosioøkonomiske forskjeller.

Tradisjonelle markeder, gateselgere og moderne supermarkeder selger ferske råvarer i alle områder av Chandigarh, og basert på mine studier ser det ut til at tradisjonelle utsalgssteder har den laveste forekomsten av råvarer som er kontaminert med parasitter i forhold til de moderne. Dette kan representere et større bilde av et utviklingsland som etterligner handelsformen i utviklede land, men med en infrastruktur som kanskje ikke er klar til å håndtere disse endringene enda.

Som i mange indiske byer er vannmangel og vannkvalitet et vanlig problem i Chandigarh. Kontaminert vann ser ut til å ramme befolkningen som bor i områder med høyere tetthet og lavere sosioøkonomisk status, selv om lave nivåer av parasitter ble funnet i de fleste positive prøver. Det er verdt å merke seg at en prøve fra slumområdene, der beboerne vanligvis mottar drikkevann i transporterte tankbiler, inneholdt et stort antall *Giardia*-cyster.

Blant de undersøkte prøvene fra bakgårdsgeitene var *Giardia* en vanlig parasitt, og isolatene som ble funnet var de samme som vi vanligvis finnes hos mennesker, og avviker fra de som vanligvis blir identifisert i geiter som lever i den utviklede verden. Dette resultatet antyder at *Giardia*-smitte mellom menneske og geit er vanligere i utviklingsland, og understreker utfordringene knyttet til mangel på grunnleggende sanitære fasiliteter i denne delen av Chandigarh, som er med på å redusere barrieren for infeksjon mellom arter.

Imidlertid var situasjonen helt annerledes hos hunder som oppholder seg i offentlige parker i Chandigarh. Der ble det funnet at de fleste *Giardia*-isolatene var spesifikke for hund. Hunder blir ikke tradisjonelt holdt som kjæledyr i indisk kultur, og i særdeleshet unngås eierløse hunder. Dette kan skyldes en frykt for å bli bitt og potensiell smitte med rabies fra slike hunder. På tross av at mennesker og hunder oppholder seg i de samme parkene i Chandigarh, ser det ikke ut til at de deler ikke de samme genotyper av intestinale protozoer.

Storfe i India blir både tilbedt og forsømt, og noen storfe beveger seg rundt i gatene side om side med mennesker som også leter etter mat. Interessant nok har disse hellige skapningene ikke mange *Giardia*-isolater med zoonotisk potensial, mens subtyper av *Cryptosporidium,* som er potensielt smittsomme for mennesker, ble også funnet i kalver.

Samlet sett gir disse studiene informasjon om mulige smitteveier for *Cryptosporidium* og *Giardia*. Det ser ut til at kulturelle og sosioøkonomiske nivåer også har noe å si for smitteveier, og selv om vann- og matbårne utbrudd av kryptosporidiose og giardiase sjelden blir rapportert eller publisert fra utviklingsland, så er det et potensiale for slik smitte der, og utbrudd fra disse landene kan være grovt undervurdert og underrapportert.

सारांश (Hindi summary)

जिआर्डिया और क्रिप्टोस्पोरिडियम प्रोटोजोअन परजीवी ख़ासकर बच्चो में दस्त का प्रमुख कारण है ये महत्वपूर्ण है, लेकिन इन्हे अक्सर नजरादाज किया जाता है, ये विशेष रूप से विकासशील देशो में सार्वजनिक स्वास्थ्य के लिए खतरा हैं। वे बड़े तोर पर मानव और पशु बीमारी का कारण बन सकते हैं, और दोनों प्रोटोजोआ में ऐसी प्रजातियां शामिल हैं जो मेजबान प्रजातियों की एक विस्तृत श्रृंखला को संक्रमित करने में सक्षम हैं, और मानव-पशु सीमाओं को पार करने के लिए अच्छी तरह से अनुकूल हैं। दोनों परजीवी के मजबूत ट्रांसमिशन चरण, उनके उच्च उत्सर्जन दर और कम संक्रामक पीने के पानी और ताजा उपज के संदूषण के माध्यम से, साथ ही सीधे सीधे संचरित हो सकते हैं।

इन तथ्यों के बावजूद, विकासशील देशों में क्रिप्टोस्पोरिडियोसिस और जिआर्डियासिस की घटनाओं और प्रकोपों पर कम रिपोर्टें हैं, जहां पानी की आपूर्ति की निगरानी नहीं है, ताजा उपज श्रृंखला ठीक से निगरानी नहीं की जाती है, और पशु विकासशील देशों की तुलना में कम प्रतिबंध के साथ घूमते हैं, जिससे मानव-पशु की सीमाएं फीकी होती हैं, इसके लिए कई कारण हैं, इसमे शायद निदान संबंधी कठिनाइयों, रिपोर्टिंग की कमी और जांच की अनुपस्थिति शामिल है; इससे यह संभावना है कि ये संक्रमण विकसित देशों में अधिक बार होते हैं।

इस थीसिस में एक प्रायोगिक भाग और एक सर्वेक्षण भाग शामिल है। प्रयोगात्मक भाग को स्वास्थ्य पर ध्यान केंद्रित किया गया है, जहां महंगे तरीकों को सस्ता कर के क्राप्टोस्पोरिडियम और जिआर्डिया से संदूषित ताजे उपज और पीने के पानी के विश्लेषण के लिए योग्य बनाया गया। इसके अलावा, प्रयोगात्मक रूप से दूषित ताजा उपज पर जिआर्डिया और क्रिप्टोस्पोरिडियम के संक्रामक चरणों का अस्तित्व मूल्यांकन किया गया, जिआर्डिया कोशिकाएं प्रशीतित तापमान से कमरे के तापमान पर संग्रहीत होने में कम सक्षम थीं, जबकि क्रिप्टोस्पोरिडियम ऑओसिस्ट्स दोनों प्रशीतित और कमरे के

तापमान पर बची हुई थी , यह आंशिक रूप से गिर्डियासिस के कुछ प्रलेखित खाद्यजनित प्रकोपों की व्याख्या कर सकता है।

मैने उत्तरी भारत के एक शहर चंडीगढ़ में इन प्रोटोजोन्स के महामारी विज्ञान, साथ ही घटना और प्रसार की जांच करने वाले पांच सर्वेक्षण अध्ययनों को भी प्रस्तुत किया है चंडीगढ़ में एक संरचित परिवेश और एक अपेक्षाकृत अच्छी तरह से विकसित बुनियादी ढांचा है, हालांकि शहर को भी अनेक समस्याओं का सामना करना पड़ रहा है जो विकासशील देशों में कई स्थितियों में करना पड़ सकता है, जैसेकि अधिक जनसंख्या, झुग्गी क्षेत्रों, खराब पानी की गुणवत्ता और पहुंच, सीवेज को साफ करना , स्वच्छता रखने मे क़ठिनाइयाँ, शौचालय की उपलब्धता के स्तर पर, आवारा पशुओं को घूमते हुए, और मानव और पशुओं दोनों के बीच विशाल सांस्कृतिक और सामाजिक-आर्थिक विभाजन पाया गया है

पारंपरिक बाजार और सड़क विक्रेताओं के साथ ही साथ आधुनिक सुपरमार्केट, चंडीगढ़ के सभी क्षेत्रों में ताजा उपज बेचते हैं, और ये देखा गया कि पारंपरिक रिटेल आउटलेट्स में आधुनिक सुपरमार्किट की तुलना में परजीवी के साथ प्रदूषित सबसे कम उत्पादन होता है। यह एक विकासशील देश का प्रतिबिंब है जो व्यापार के संदर्भ में विकसित होने की नकल करता है, लेकिन बुनियादी ढांचे शायद इन परिवर्तनों को संभाल करने के लिए तैयार नहीं है।

कई भारतीय शहरों की तरह, चंडीगढ़ में भी, पानी की कमी एक आम समस्या है। दूषित पानी उच्च घनत्व वाले क्षेत्रों में रहने वाले आबादी को प्रभावित करता है, हालांकि सबसे सकारात्मक नमूने में परजीवी की संख्या कम पाई गयी विशेष रूप से, झुग्गी क्षेत्र से एक पानी के नमूने मे सबसे ज्यादा जियर्डिया सिसट प्राप्त हुई, जहां निवासियों को आमतौर पर परिवहन के टैंकों में अपने पेयजल प्राप्त होता हैं

घरो मे रखी बकरियो में जिआर्डिया आम पाया गया, इसमे पाए गये आइसोलेट मनुष्यों में पाए जाने वाले आइसोलेट के समान थे, प्ररंतु ये विकसित देशों में रहने वाले बकरियों के आइसोलेट्स से अलग थे इस परिणाम से पता चलता है कि इन मनुष्यों और बकरी आबादी में बुनियादी स्वच्छता सुविधाओं की

कमी के कारण इस स्थिति में पश्चिमी "बकरी बकरी" संचरण चक्र की बजाय एक "मानव बकरी", चक्र चलता है

हालांकि, चंडीगढ़ में मनोरंजक पार्कों में घूमने वाले कुत्तों में स्थिति पूरी तरह से अलग थी, जहां जिआर्डिया अलग-अलग इलाकों में डिब्बे-विशिष्ट होते थे। कुत्तों को पारंपरिक रूप से भारतीय संस्कृति में पालतू जानवर के रूप में नहीं देखा जाता है, और विशेष रूप से आवारा कुत्तों से बचा जाता है। इसका कारण कुतें द्वारा काटे जाना और रेबीज की संभावना का डर हो सकता है। इस प्रकार, भले ही मानव और कुत्तों चंडीगढ़ में एक ही पार्क में घूमते हैं, वे एक ही तरह के आंतों के प्रोटोजोऑन को साझा नहीं करते हैं।

भारत में गाय की पूजा की जाती है और नज़रअंदाज भी किया जाता है, और कुछ पशु अपने भोजन की तलाश मे मानवो के साथ सड़को पर घूमती हैं, दिलचस्प बात यह है कि इन पशुयो मे पाए गये जिआर्डिया की ज़ोंटोटिक क्षमता नहीं थी, लेकिन चंडीगढ़ में मनुष्यों में पाए गये क्रिप्टोस्पोरिडियम के उपप्रकार बछड़ों में भी पाए गए।

एक साथ यह कह सकते है क़ि इस अध्ययनों में क्राप्टोस्पोरिडियम और जिआर्डिया के संभावित ट्रांसमिशन पथ के बारे में जानकारी दी गई है। ऐसा लगता है कि सांस्कृतिक और सामाजिक आर्थिक स्तर ट्रांसमिशन मार्गों पर भी एक भूमिका निभाते हैं, और यद्यपि विकासशील देशो मे क्रिप्टोस्पोरिडियोसिस और गियारडायसिस के जलजनित और भोजनजन्य प्रकोप शायद ही कभी रिपोर्टिंग या प्रकाशित होते हैं या यह कह सकते है ये प्रकोप बेहद कम अनुमानित और अंतर्निहित हैं।

1. Introduction

1.1 Background

Human and animal populations in developing countries are under a constant threat from diseases. This may be due to a combination of poor infrastructure, poor knowledge, poor health facilities, and poor management, which may all boil down to poverty itself. In addition, many pathogens seem to thrive in the climate of these parts of the world. Transmission is exacerbated by the lack of infrastructure, including basic sanitation and water treatments, along with lack of diagnosis and treatments. *Cryptosporidium* and *Giardia* are only two of a plethora of pathogens causing disease in these settings, but, in contrast to other well-known disease-causing microorganisms, they have not been the focus of the attention they deserve as severe debilitating agents. Their biology makes them suitable for both water- and food-borne transmission, and many outbreaks thereof have been described in the developing countries, along with follow-up studies on their long-term health effects on those affected. However, in developing countries such outbreaks are very seldom studied and published, and do not garner the same attention or follow-up. Some species and genotypes of these parasites are also zoonotic, which gives them the ability to spread across species, and this potential has been widely studied and described in industrialised countries, where the animal-human interface is more separated, especially in the context of farm animals, and proper management and hygiene is part of the daily routine. Relatively little of this potential and different ways of transmission have been described from developing parts of the world.

The diseases that are the subject to most attention tend to be those with acute symptoms and high morbidity and mortality, and are usually also those that create dramatic headlines for the media in the developed parts of the world.

Diarrhoea might not be the most eye – catching topic for the general public, but for many children in the developing areas of the world it is a common headline every day. In fact, diarrhoea accounts for 4% of all deaths worldwide, and mostly affect children in developing countries (WHO, 2000)

Furthermore, the standard methods for identifying protozoans in drinking water and vegetables that are applied in developed countries, are currently too expensive for use in settings where resources are already highly stretched, and the application of such methods to routine laboratories are of questionable value with respect to source tracking, as incidences and outbreaks occurs against a backdrop of high prevalence. Interfaces between humans and animals are not so demarcated in such settings, and families may even share household with their livestock under poor sanitary conditions. The risk of infection with intestinal parasitic protozoans can be avoided by the implementation of proper hygiene, appropriate livestock management, availability of foods that are safe to consume, and clean water. These are facilities the population in developed countries may take for granted, and even consider a human right, but for many inhabitants of this globe it is not safe to be thirsty.

1.2 *Giardia* and *Cryptosporidium* in developing countries

Cryptosporidium as the pathogen of surprise

A multicentre study examined the underlying causes of childhood diarrhoea in different developing regions spread across the globe, and some of the results were astonishing. There were only five major causative agents, and amongst those there was a surprise for most doctors, epidemiologists, and parasitologists: the protozoan *Cryptosporidium* was ranked as being of second highest importance for causing moderate to severe diarrhoea in toddlers. Cryptosporidiosis has previously been mostly known to cause generally self-limiting diarrhoea, sometimes including nausea, vomiting and fever, which usually resolves within a week in normally healthy people, but also may last for a month or more. It has largely been considered a problem for the immunosuppressed population, due to the absence of effective treatment. The diarrhoeal disease was found to have lasting health repercussions after the acute phase of infection, manifested as increased mortality risk and significant growth delay. Although the study sites were spread over developing regions across the globe, these findings were largely consistent. The study concluded that changing the way diarrhoeal disease is cared for, by longer-term monitoring and rehabilitation, could improve health and survival, and emphasised that developing new tools for targeting the top

pathogens, especially *Cryptosporidium,* for which few measures of treatment currently exist, is essential. It was also the only pathogen with an association with elevated mortality (Kotloff et al. 2013). This was further established by a longitudinal study, giving a high Attributed Factor (The proportion of cases or deaths from a disease which could be avoided if exposure was eliminated) to *Cryptosporidium* spp. in the first year of life, and an association between *Cryptosporidium* and more severe diarrhoea.

The study by Kotloff et al., (2013) focused on acute and moderate to severe diarrhoea, but non-severe diarrhoea episodes are also important to the public health due to their high prevalence and association with stunted growth and development, and even elevated mortality in developing regions. A study documenting the broad range of pathogens (up to 25 pathogens in second year of life) associated with any severity in low- and middle income countries suggested that causes of community diarrhoea are diverse, and although single targeted pathogen interventions may have an important role in the reduction of the burden of severe diarrhoeal disease, it may not have a substantial impact on the total diarrhoeal incidence in a community (Platts-Mills et al. 2015). *Giardia* was the fourth most frequently detected pathogen on an overall basis, and the results regarding moderate to severe diarrhoea were consistent with the findings of Kotloff et al.(2013).

These two important studies concluded that:

- There are specific pathogens causing a high burden of moderate to severe diarrhoea in children in developing countries.
- There is a plethora of pathogens, with a questionable attributable factor, circulating at all times in developing communities, and which contribute to occasional disease and general failure to thrive amongst children.

Not so severe right then and there, but there`s more to *Giardia* than just to adhere

Giardia is also a common aetiological agent of diarrhoea. As many as 280 million cases occur per year, and severe symptoms may be persistent and sometimes even life-threatening for the immunocompromised and aged population, as well as infants (Lane & Lloyd 2002). Nonetheless, although perhaps not contributing substantially to severe disease, *Giardia* infections in early life may be associated with stunted growth and development (Donowitz et al. 2016). A meta–analysis concluded that *Giardia* generally does not cause acute diarrhoea in children from developing regions, but is associated with persistent diarrhoea (Muhsen & Levine 2012), and has been connected to long-term sequelae such as irritable bowel syndrome (IBS), (Robertson et al. 2006), pruritis and urticaria (Prieto-Lastra et al. 2006), uveitis, (Gelfer et al. 1984), food allergies, (Di Prisco et al. 1998; Hanevik et al. 2009) and synovitis (Letts et al. 1998). All these sequelae have been studied in developed countries with follow-up of patients. As *Giardia* is one of the most widespread pathogens in developing countries, the number of people suffering from long-term effects could represent a large part of the work-force, children attending school, and those already immunosuppressed - all trying to survive on already scarce resources far from, be it economically or physically or both, the nearest health care facility.

These infections are normally perceived as having a short duration and few complications, although *Giardia* and *Cryptosporidium* infection in infants and children are in fact associated with poor cognitive functions and failure to thrive (Berkman et al. 2002). This fact emphasizes the need for these parasites to step into the limelight of important pathogens.

Sources of infection

Although both *Giardia* and *Cryptosporidium* are infectious immediately after excretion, and thus direct faecal-oral transmission is probably the most common route of transmission, several occurrences of waterborne outbreaks of cryptosporidiosis and giardiasis have been reported and published, and the vast majority of these outbreaks have been described from developed countries.

The WHO estimated in 2004 that 88% of diarrhoeal deaths are due to unsafe water supply and poor hygiene and sanitation, more than 99% of these deaths occur in developing countries (WHO 2004), and, furthermore, that about 84% of these occur in children (WHO 2009). Safe drinking water remains inaccessible for about 1.1 billion people in the world,

and, at any given time, about half the population in the developing world is suffering from diseases associated with water supply and sanitation (Gadgil 1998). Since 1990, 2.6 billion people have gained access to improved drinking water sources, still 663 million people are without. As both *Cryptosporidium* and *Giardia* are common waterborne diseases, and when considering that 1.8 billion people use a source of drinking water which is faecally contaminated (UNDP 2017), contaminated drinking water may represent a significant source of these infections.

The WHO also gives guidelines on the extent to which drinking water initiatives can reduce these infections by means of improving sanitation and points of disinfection, and also by improving the water supply itself (WHO 2004). Although these are worthy initiatives, it seems that the sources of contamination are somehow overlooked, and the focus is on treating already contaminated water, and how to treat humans who are already infected. Although many studies have documented the prevalence and occurrence of different parasitic infections, few of them included efforts to identify the sources of infection and how the patients acquired the disease. One of the aims of this thesis is to search for clues regarding these sources and give some suggestions on how they may be eliminated, trying to shift the focus of these diseases to prevention before treatment, by tracking the possible sources of infection and providing more affordable methods to detect these sources.

Contamination from livestock has been incriminated as the source of waterborne outbreaks of cryptosporidiosis and giardiasis on various occasions. Indeed, close contact with farm animals is known to increase the risk of acquiring infection with *Cryptosporidium*, and outbreaks of cryptosporidiosis among veterinary students are widely reported. However, among the more recent outbreaks of waterborne cryptosporidiosis and giardiasis, molecular analyses tend to indicate that contamination of the supply by human sewage is often the more likely culprit.

Vegetables and other fresh produce have also been noted as potential vehicles of infection for *Cryptosporidium* and *Giardia*, although to a lesser extent than the human-to-human and waterborne route. An expert elicitation found that the proportion of DALYs contributed by the foodborne route for *Cryptosporidium* was 4 on a global scale with a dispersion from 6 in the South-East Asian region and 0.2 for Europe. The corresponding numbers for *Giardia* was 0.3 with a dispersion from 0.4 in the Western pacific region and 0.03 in Europe (Kirk et al.

2015). In relation to other routes of contamination, the proportion of illnesses caused by *Cryptosporidium* through different exposure routes was 0.37 for water and 0.10 for food in South East Asia, and the same proportions were 0.38 and 0.10 for Europe. For *Giardia*, the proportions for waterborne giardiasis was 0.35, and foodborne giardiasis was 0.13 for South-East Asia, while in Europe the equivalent figures were estimated to be 0.32 and 0.11, respectively (Hald et al. 2016).

Recent initiatives are being made to ensure clean and safe water where it is most needed, for example through the Sustainable Development Goals formed in 2016, and one of their missions is specifically named "Clean water and sanitation", with an ambitious goal of ensuring universal access to safe and affordable drinking water for all.

With these findings in mind, this projects aim was to find sources and occurrences of *Cryptosporidium* and *Giardia* in potable water sources, Vegetables commonly consumed raw, and common livestock, stray animals and pets kept in Chandigarh, a city in northern India.

1.3 General presentation of *Cryptosporidium* and *Giardia*

1.3.1 *Cryptosporidium* taxonomy, species and life cyle

Cryptosporidium (Subphylum Apicomplexa) is a genus of protozoan parasites infecting the microvilli of epithelial cells in the digestive, and sometimes the respiratory tract, of humans and animals. *Cryptosporidium* has a wide host range, which includes at least 155 mammalian species (Fayer 2004), as well as reptiles, birds, amphibians and fish.

Currently, 27 species of *Cryptosporidium* and over 40 genotypes are recognized (Ng et al, 2011). The majority of human infections are caused by *Cryptosporidium hominis* and *C. parvum*. It has been proposed that the name *Cryptosporidium parvum* should be changed to *Cryptosporidium pestis* (Slapeta, 2006), but this new nomenclature has not been widely accepted due to lack of taxonomic description (Xiao et al, 2012). In addition to *C. hominis and C. parvum, C. meleagridis, C. felis, C. canis, C. suis, C. muris, C. andersoni, C. ubiquitum, C. viatorum, C. cuniculus* and the C*ryptosporidium* horse, skunk and chipmunk I genotypes have also been detected in stools of immunocompetent and immunocompromised humans (Fayer, 2010; Xiao, 2010; Elwin et al., 2012; Kvac et al., 2013). *C. parvum* is the major zoonotic species causing cryptosporidiosis in livestock, and this species, in particular, makes a substantial contribution to environmental contamination due to high excretion rates (Smith et al, 1995). Various different molecular tools have been used in the differentiation of *Cryptosporidium* species/genotypes and of subtypes among some species such as *C. parvum* and *C. hominis*.

Table 1. Currently recognized species of *Cryptosporidium*, with major and minor host ranges and location within the host (Elwin et al. 2012; Fayer 2010; Jirku et al. 2008; Kváč et al. 2013; Ren et al. 2012; Robinson et al. 2010; Smith et al. 2005).

Species	Major hosts	Minor hosts	Location
C. andersoni	Cattle	Sheep, humans	Abomasum/small intestine
C. baileyi	Poultry	Quails, ostriches, ducks	Small intestine
C. bovis	Cattle	Sheep	Small intestine
C. canis	Dogs	Humans	Small intestine
C. cuniculus[6]	Rabbit	Humans	Small intestine
C. fayeri	Red kangaroo	Not known	Small intestine
C. fragile	Toads	Amphibians	Stomach
C. felis	Cats	Humans, cattle	Small intestine
C. galli	Finches, chicken	Not known	Proventriculus
C. hominis	Humans	Dugong, sheep	Small intestine
C. macropodum	Eastern grey kangaroo	Not known	Stomach
C. meleagridis	Turkey, human	Parrots	Small intestine
C. molnari	Fish	Not known	Stomach and intestine
C. muris	Rodents	Humans, hyrax, goat	Stomach
C. parvum	Cattle, human	Deer, mice and pigs	Small intestine
C. ryanae[1]	Cattle	Not known	Not known
C. scrofarum[4]	Pigs		Small intestine
C. scophthalmi	Fish	Not known	Stomach and intestine
C. serpentis	Lizards, snakes	Not known	Stomach
C. suis	Pigs	Humans	Small and large intestine
C. tyzzeri[7]	Mouse	Humans, ruminants	Small intestine
C. ubiquitum[2]	Cattle	Humans, sheep, deer	Small intestine
C. varanii[5]	Lizards	Snakes	Stomach and intestine
C. viatorum	Humans	Not known	Not known
C. wrairi	Guinea pigs	Not known	Small intestine
C. xiaoi[3]	Sheep	Yak, goat	Not known

[1] Formerly known as deer-like genotype, [2] Formerly known as cervine genotype, [3] Formerly known as C.bovis-like genotype, [4] Formerly known as pig genotype II, [5] Formerly known as C. saurophilum, [6] Formerly known as rabbit genotype, [7] Formerly known as mouse genotype I

Cryptosporidium genotypes and subtypes, and their zoonotic potential

Molecular tools have been extensively used to characterize the transmission of human cryptosporidiosis. Five *Cryptosporidium* spp are responsible for most infections, namely *C. hominis, C. parvum, C. meleagridis, C. canis* and *C. felis.* In developing countries, *C. hominis* is the causative agent for about 70% of infections, while *C. parvum* accounts for 10-20%. Some differences have been found in endemic areas in proportion of infection attributable to species, for example has *C. meleagridis* been found as the main causative agent in some areas. Subtyping results suggests that there is high genetic heterogeneity in *C. hominis* in developing countries, and geographical segregation of both *C. hominis* and *C. parvum* subtypes (Xiao 2009). In Europe, both *C. hominis* and *C. parvum* are most common and responsible for most human infections (Bajer et al. 2008; Chalmers et al. 2009; Leoni et al. 2006; Llorente et al. 2007; Nichols et al. 2006; Savin et al. 2008; Wielinga et al. 2008; Wolska-Kusnierz et al. 2007; Zintl et al. 2009; Šoba et al. 2006), while in the middle east, *C. parvum* is the dominant species infecting humans (Al-Brikan et al. 2008; Meamar et al. 2007; Pirestani et al. 2008; Sulaiman et al. 2005; Tamer et al. 2007). Thus, there is a vast diversity of cryptosporidiosis transmission, highlighting the need for more extensive studies of cryptosporidiosis epidemiology in diverse areas, including several socioeconomic strata and environmental conditions.

The combination of subtyping and conventional epidemiological tools can improve the assessment of the disease burden attributable to zoonotic transmission. A large number of studies have been conducted to subtype *C. parvum* in farm animals, with the focus on calves as infection is largely associated with younger animals. Most subtyping studies have used gp60 sequence analysis, and have been done in developed countries. The results have shown that calves are commonly infected with subtypes in the IIa family, with the subtype IIaA15G2R1 being especially common (Xiao 2010). Although have several subtypes been found to be more regionally distributed, and IId subtypes have been found to be especially common in lambs and goat kids in Spain, though the IIa subtypes are more common in calves in the same area (Quilez et al. 2008a; Quílez et al. 2008b).

Many of the common bovine IIa family subtypes in North America, Europe and Australia are also dominant *C. parvum* subtypes in humans in these areas(Alves et al. 2006; Feltus et al. 2006; Jex et al. 2007; Jex et al. 2008; Ng et al. 2008; O'Brien et al. 2008; Soba & Logar 2008;

Waldron et al. 2009; Zintl et al. 2009). IId is another major zoonotic genotype family reported in Europe, Asia and Africa (Amer et al. 2013; Imre et al. 2013; Insulander et al. 2013; Iqbal et al. 2012; Wang et al. 2011), but this family has never been found in humans in the United States and Canada, where they also seem to be absent in calves (Xiao 2010).

These findings suggest that there are differences in the role of zoonotic transmission of *C. parvum* among geographic areas, and even the zoonotic implications of some subfamilies have been questioned. Studies from Portugal and Slovenia showed that the genetic diversity of *C.parvum* was much higher in humans than in calves, and subfamily IIc was not even found in animals (Alves et al. 2006; Soba & Logar 2008)

Results of multi-locus genotyping studies have further supported the occurrence of anthroponotic *C. parvum* (Xiao 2010). Thus, a significant fraction of *C. parvum* infections may not have originated from a ruminant reservoir.

Cryptosporidium parvum transmission in developing countries appears largely anthroponotic, as the most common subtype family is IIc, and has even been found to be the only prevailing subtype in countries such as Lima, Peru and Jamaica, and studies from India, Uganda, Malawi and Kenya have found some unusual *C. parvum* subtype families such as IIb and IIe in humans, which have never been found in animals anywhere (Akiyoshi et al. 2006; Cama et al. 2003; Cama et al. 2008; Gatei et al. 2008; Muthusamy et al. 2006; Savioli et al. 2006).

These anthroponotic speculations from developing countries still needs support of results from animal studies, as gp60 subtyping has been done on only a few *C. parvum* isolates from these areas.

Life Cycle of *Cryptosporidium*

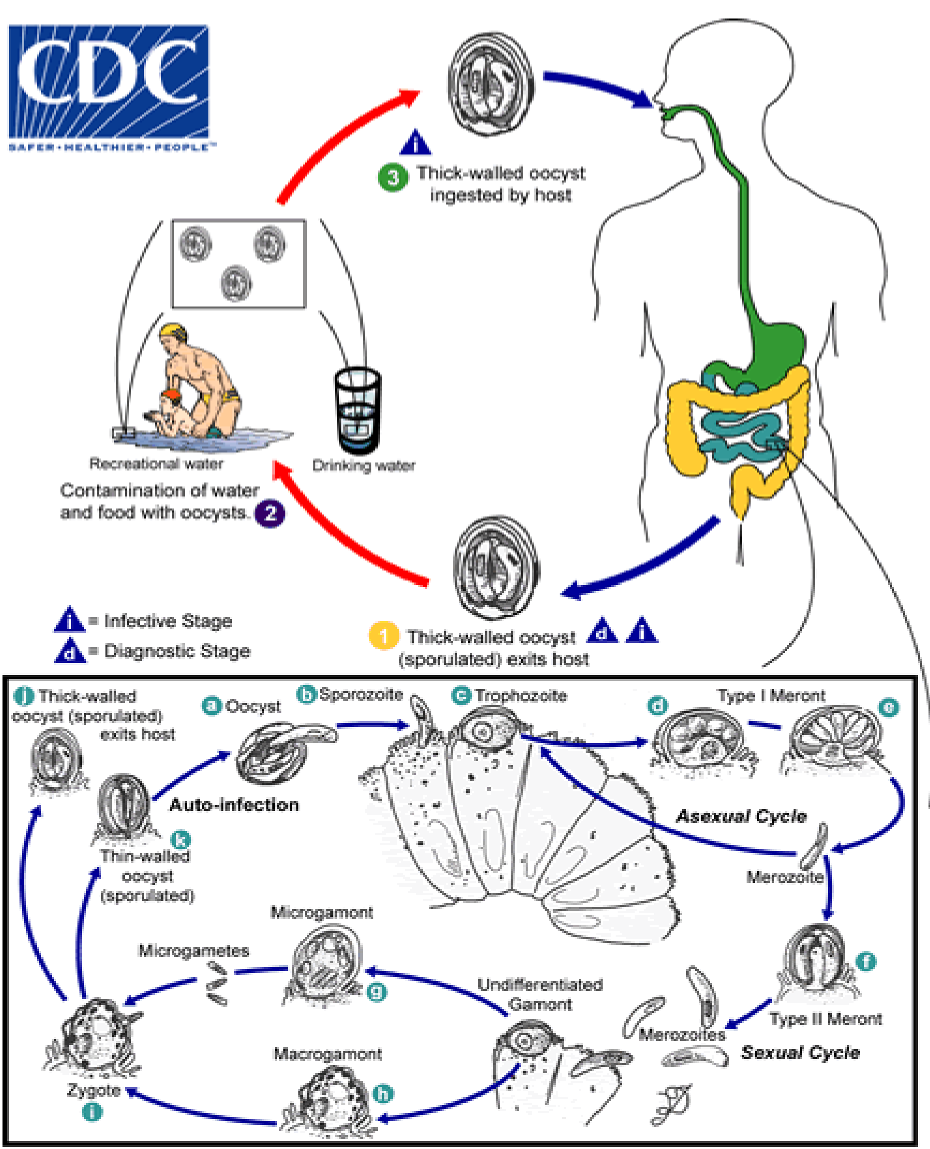


Figure 1. Life cycle of *Cryptosporidium* (CDC 2016).

Cryptosporidium has a complex life cycle, including both sexual and asexual phases. The cycle can be completed within three to five days. Infectious dose for humans can be as low as 1-5 oocysts (Guerrant 1997), although this is highly dependent on the species and strain of *Cryptosporidium*, and the immune status of the host, with immunodeficient persons being more susceptible (Goldstein et al. 1996). The median infectious dose for one strain of *C.*

parvum was demonstrated to be 132 oocysts in healthy adult volunteers (DuPont et al. 1995).

After the oocysts reach the small intestine, they excyst and four motile sporozoites leave the oocyst (Hijjawi et al. 2002; Smith et al. 2005), and subsequently infect the epithelial cells. In the cells, the sporozoites reside in a parasitophorous vacuole between the cell membrane and cell cytoplasm. Inside this epicellular location, the sporozoites form trophozoites that undergo asexual development, with two successive generations of merogony, resulting in the formation of meronts. Merozoites develop into sexual developmental stages known as the micro- and macrogametes. The microgametes are released from the host cells and penetrate cells harbouring macrogametes, and their fusion results in the formation of a zygote, which develops into an oocyst with a resistant oocyst wall. The oocysts are approximately spherical and measures between 4-5 µm in diameter. Most (80%) of the oocysts have a thick wall and are excreted with faeces, and represent the environmentally resistant stage of the parasite, and are immediately infectious. The remaining 20% are thin-walled oocysts, and are believed to cause autoinfection through recycling of sporozoites from ruptured thin-walled oocysts (Hijjawi et al. 2001).

Diagnosis and detection of cryptosporidiosis

Although first described in 1907, *Cryptosporidium* was not considered as a pathogen in livestock until 50 years later, when it was recognized to cause morbidity and mortality in young turkeys in 1955, and it was first described as a disease-causing agent in humans 20 years thereafter, in 1976, when the first two cases were described histologically (Meisel et al. 1976; Nime et al. 1976). This delay in recognition of *Cryptosporidium* as a pathogen, may partly be due to the lack of effective methodologies at that time which were able to detect parasites in clinical samples. The AIDS pandemic in the 1980s, in which *Cryptosporidium* was found to be a concomitant, often fatal, pathogen, along with several large waterborne outbreaks, brought the realisation of the public health significance of this parasite.

Oocyst morphology

Figure 2. Intact and ruptured *Cryptosporidium* oocyst. (Picture acknowledgement, Birgitte Kasin Hønsvall)

Sporulated oocysts are smooth, colourless, spherical or ovoid and contains four elongated sporozoites, which are characterized by their comma-shape, and a residual body. These contents can be difficult to distinguish by light microscopy. Their morphometry can be helpful in distinguishing oocysts from other microscopic artefacts, but is not sufficient to distinguish species. In diagnostics, it is common to use staining to identify oocysts in faecal and environmental samples.

1.3.2 *Giardia* taxonomy, species and life cycle

The genus *Giardia* belongs to the kingdom Excavata, clade Fornicata and order Diplomonadida. *Giardia* comprises 6 species, distinguished on the basis of light- and electron microscopy of the trophozoite (Adam 2001), of which five are isolates from birds, amphibians, mice and voles, and the sixth species, *Giardia duodenalis* (syn. *G. lamblia*, syn. *G. intestinalis*) is a complex containing strains isolated from a large range of mammalian hosts grouped into a single species by (Filice 1952).

Giardia genotypes and their zoonotic potential

Genetic analysis has so far revealed eight distinct assemblages within the species complex *G. duodenalis*, named with letters from A to H.

Assemblages A and B cause infection in humans, as well as being reported from a range of other mammals, whereas the remaining assemblages are more restricted in their host range; Assemblages C and D are found in canids, E in livestock or ungulates, F in cats, G in rodents, and H in pinnipeds. The genetic distance between assemblages of *Giardia duodenalis* is of the same level as the other *Giardia* species, and new individual species names have been

proposed for the different assemblages. However, this further division into seven species and new nomenclature is not a widely accepted and will not be further elaborated in this thesis. Table 2 summarizes the host range for the established *Giardia* species (Feng & Xiao 2011).

Table 2: The established *Giardia* species and *G. duodenalis* assemblages with their corresponding host range (Lasek-Nesselquist et al. 2010; Xiao & Fayer 2008).

Species		Major host(s)
G. duodenalis species complex:		
	Assemblage A	Humans, non-human primates (NHP), ruminants, pigs, horses, canines, felines, rodents and other mammals
	Assemblage B	Humans, NHP, ruminants, canines, horses, rabbits and rodents
	Assemblages C and D	Canids
	Assemblage E	Ungulates and pigs
	Assemblage F	Cats
	Assemblage G	Mice and rats
	Assemblage H	Pinnipeds
G. agilis		Amphibians
G. ardeae		Birds
G. psittaci		Budgerigar
G. microti		Rodents
G. muris		Rodents
G. varani		Lizards

Life cycle of *Giardia*

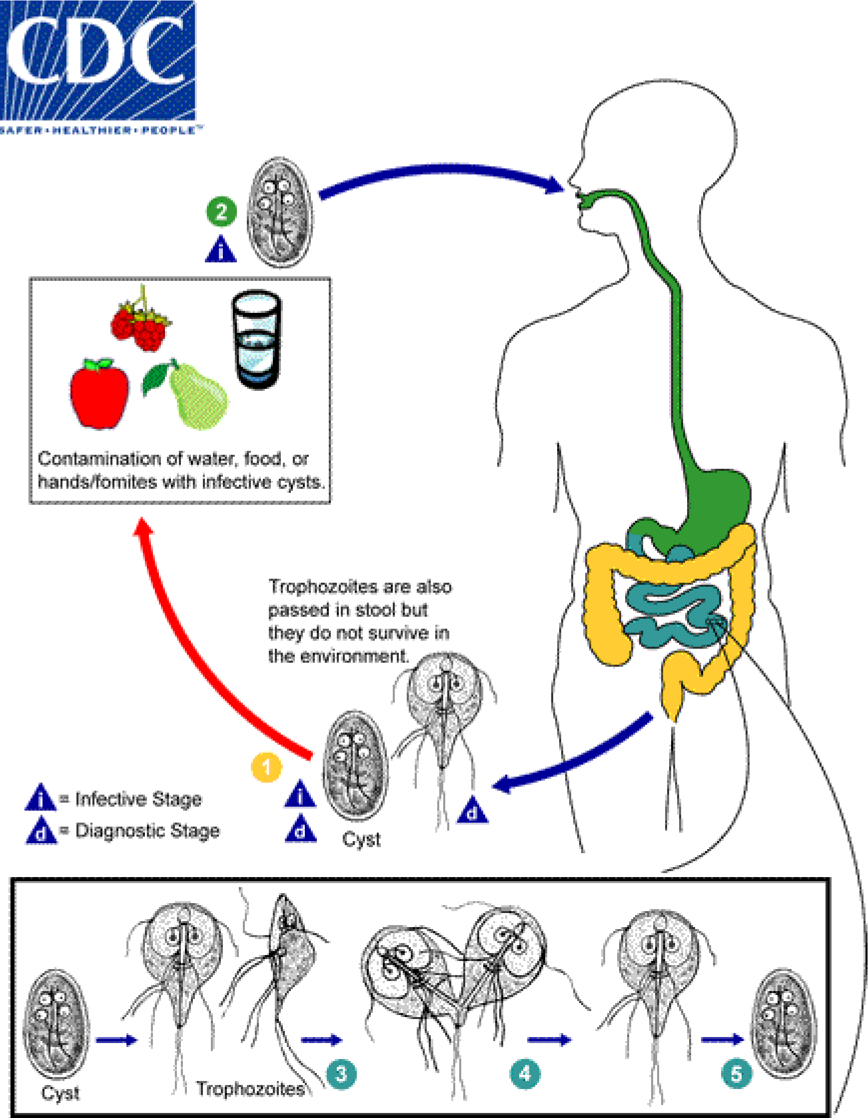


Figure 3. Life cycle of *Giardia* (CDC 2017).

The life cycle of *Giardia* is quite simple and consists of two life stages:

I: a dormant and infective cyst, resistant to many stressors and II: an active, replicating trophozoite colonizing the small intestine of its host and causing, in most, but not all, cases, clinical signs. The cycle can be completed within 12 to 19 days (Jokipii et al. 1985). The infectious dose for humans may be as low as 10 cysts (Ortega & Adam 1997), and the median infectious dose is between 10 and 100 cysts (Cooper & Olivieri 1998).

After ingestion, two motile, flagellated trophozoites emerge from the cyst when reaching the small intestine, with excystation triggered by the intestinal environment. The trophozoites attach themselves to the epithelial cells by their adhesive discs and colonise the small intestine. The trophozoites replicate through repeated binary fission, and, unlike *Cryptosporidium* sporozoites, *Giardia* trophozoites are not invasive, although the suction force from the adhesive discs may damage the microvilli of the small intestine. Exposure to biliary salts leads to the encystation of trophozoites in the jejunal part of the small intestine, forming elliptical, 8-12 µm long and 7-10 µm wide cysts, which are excreted with the faeces, and are immediately infectious.

Diagnosis and detection of giardiasis

The first description of *Giardia* was by van Leeuwenhoek in 1677, although clinical interest in this protozoan species began only 40 years ago with the isolation of *Giardia* from mammalian, avian, and amphibian hosts, and it was only in the late 1970s that *Giardia* was recognized to cause disease (Kreier 1978). The parasite was added to the World Health Organizations list of parasitic pathogens as late as in 1981 (WHO 1981).

Cyst and trophozoite morphology

The trophozoite form of *Giardia* has a characteristic tear-shape, with a bi-radial symmetry. Its ventral disc, used for attachment to epithelial cells, is composed of a single layer of microtubules. Each trophozoite has four pairs of flagella that are situated anteriorly, posterior-laterally, caudally and ventrally, and are used for motility within the host intestine. The cytoskeleton makes up the unique structure of these flagella and ventral disc, as well as the median body.

The cyst is the infective life stage of *Giardia*. They are already in an infective state when excreted with the host feces. The cysts measure between 5 to 10µm, have an ellipsoid form and carry between two to four nuclei, depending on whether they contain one or two trophozoites.

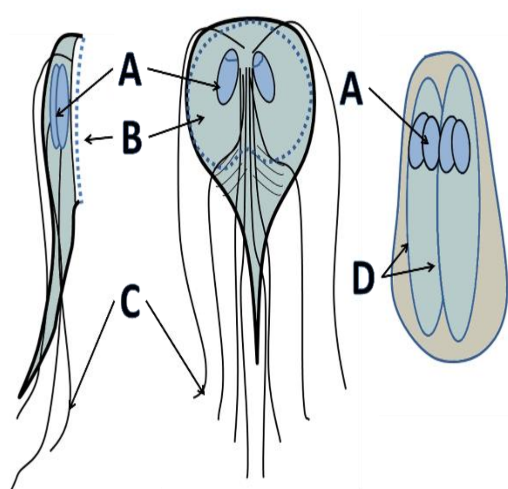


Figure 4. Trophozoite, lateral and front/back view, and cyst with two trophozoites

(Picture acknowledgement, Kristoffer R. Tysnes)

A: Nuclei

B: Adhesive disc

C: Flagella

D: Trophozoites

1.3.3 Detection and diagnosis of *Cryptosporidium* and *Giardia*

Infection with these protozoans may be identified from a faecal sample by detecting (oo)cysts, their antigens, or by detection of their DNA. Of these techniques, PCR and IFAT are considered to have higher sensitivities than antigen detection or direct microscopy, although several antigen detection methods utilizing enzyme immunoassay also have high sensitivities and specificity; the *Cryptosporidium* lateral flow (TechLab, Inc., Blacksburg, VA) immunochromatographic assay which has been developed within the last few years has also had very good reports (Fleece et al. 2016). Detection of host antibodies (serology) may also be used for determination of exposure.

Microscopy

In the developing world, wet-mount faecal preparations after concentration remains the main tool used to diagnose intestinal parasitic disease by ova and parasite examination (Ryan et al. 2017). This is a technique based on direct visualisation and is useful for rapid diagnosis in cases where large numbers of parasites are excreted.

Detection of *Giardia* cysts and *Cryptosporidium* oocysts by microscopy can also be improved by using flotation prior to examinations using Sheather's sugar, zinc sulphate, or saturated sodium chloride solutions. The use of solutions of different densities separates (oo)cysts from the rest of the faecal debris, and by reducing the background debris, makes them easier to identify. An alternative approach for removing debris, and which is more commonly used in human parasitology labs than veterinary ones, is use of formol-ether or ethyl acetate

sedimentation. This removes or decreases the fat in the samples, thereby aiding identification. As cryptosporidiosis, and, more commonly giardiasis, are associated with steatorrhoea, this may be a useful adjunct for clinical specimens. However, losses of oocysts and cysts in the faecal plug may be expected.

Stains may be added to the sample to aid in identification, as such stains are selected to highlight particular features of the parasites, thereby making them more easily distinguished against the background debris. A number of different stains are available to assist in the detection of *Cryptosporidium* oocysts in samples. Giemsa staining was the first to be used by Edward Tyzzer himself, who first described the parasite, and is now commonly used to differentiate nuclear and cytoplasmic morphology of parasites. Other stains include Romanowsky, modified Ziehl-Neelsen, auramine phenol, carbol fuchsin, potassium permanganate and safranin methylene blue. Most of the direct staining methods are cheap and easy to perform, although some such as auramine phenol require a fluorescence microscope for screening, but none of them have been reported to have sensitivities below 10^3-10^4 oocysts per gram faeces (Peeters & Villacorta 1995).

For detecting *Giardia* when using direct smears, or wet mounts, the slide can be stained with Lugol's iodine or trichrome stain (Shetty & Prabhu 1988). A widely used method for staining *Giardia* trophozoites is Giemsa staining, in which the samples containing trophozoites are washed and dried before being fixed to a microscope slide using methanol.

However, the majority of specimens do not tend to contain trophozoites, except in cases of severe diarrhoea or when endoscopy has been performed, as they are not transmission stages and die relatively rapidly in the environment.

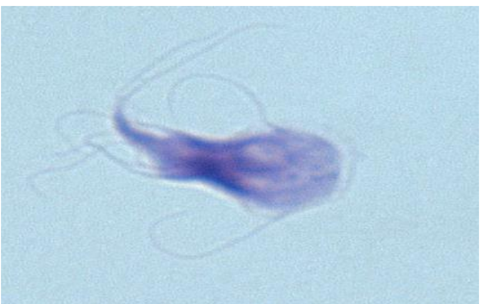


Figure 5. *Giardia* trophozoite stained with Giemsa (Picture acknowledgement, Kristoffer R. Tysnes).

There are also commercially available antibodies with fluorescent tags that bind to the parasite (oo)cyst walls, making them "glow" when viewed under a fluorescent microscope equipped with the appropriate filter blocks for the fluorophore being used. The most commonly used fluorophore is fluorescein isothiocyanate (FITC), which has excitation and emission spectrum peak wavelengths of approximately 495 nm/519 nm, giving it a green colour, but other fluorochromes can be used.

As this staining technique is dependent on both antibody binding and fluorescent detection, it is called an immunofluorescent antibody test (IFAT), and is currently considered to be the gold standard in the detection of *Cryptosporidium* oocysts and *Giardia* cysts in faeces or environmental samples, being the detection method of choice for standard methods, such as ISO 15553 (ISO, 2006) and ISO 18744 (ISO, 2016).

Some limitations in using some of the microscopy techniques are that they are labour intensive, time-consuming, can lack sensitivity and specificity, and require a high level of skill for optimal interpretation, and there is a lack of skilled technicians in both developing and developed countries (McHardy et al. 2014). In addition, IFAT not only is an expensive fluorescence microscope equipped with appropriate filter blocks essential, but the reagents necessary require to be kept in cold storage, thus diminishing their use in countries where availability of refrigeration is not always optimal.

Antigen detection techniques

Enzyme-linked immunosorbent assays (ELISA) can also also be used to detect *Cryptosporidium* and *Giardia*, and there are several commercial tests available for antigen detection. Although these tests are mainly developed for parasite species that are pathogenic for humans, and thus have unknown applicability for other species within these genera.

DNA-based techniques for detection of *Cryptosporidium* and *Giardia*

By using molecular diagnostics, it is possible to detect the presence of small amounts of parasite DNA in a sample. The code of the specific DNA can be sequenced, and information on the specific genetic structure of the isolate, which then may allow determination of the phylogeny of the isolate, and possible transmission pathways and epidemiology. Depending on the genes targeted and whether the intention is to obtain more information rather than

just detection, other aspects, such as virulence, may also be explored. These techniques are further elaborated in section 4.3.3.

1.3.4 Treatment of cryptosporidiosis and giardiasis

Treatment of cryptosporidiosis in humans

Considerable effort and resources have been directed towards trying to find an effective cure for cryptosporidiosis. Over 100 compounds have been evaluated for the treatment of cryptosporidiosis in humans, mice, and cattle, but none of them have been able to control or eliminate clinical signs or infection in all hosts (Gargala 2008), and a large number of antimicrobial drugs have been tested in both animals and humans infected with *Cryptosporidium*, with no clear evidence of effect (Mead 2002).

A meta-study assessing treatment of cryptosporidiosis in immunocompromised individuals with nitazoxanide and paramomycin found no effect on the patient groups in focus, and although the study indicated that immunocompetent patients achieved parasitological clearance after treatment with nitazoxanide, the authors concluded that there is no evidence to support the role of chemotherapeutic agents in managing cryptosporidiosis in immunocompromised individuals (Abubakar et al. 2007). From 2004, nitazoxanide was licensed by US FDA for treatment of cryptosporidiosis in all immunocompetent patients over 1 year of age (from 2002 it was only licenced for children aged 1-11 years), but it is currently not approved for treatment of cryptosporidiosis in immunodeficient persons, which are the patient group most at risk of severe infection in the United States, because placebo treatment was shown to be equally effective at treating *Cryptosporidium*-associated diarrhoea in these patients.

However, for HIV-infected patients suffering from cryptosporidiosis, intense antiretroviral therapy can lead to complete resolution of clinical symptoms and oocyst secretion(Grube et al. 1997; Miao et al. 2000), although there are no established treatment regime against *Cryptosporidium* infections in this patient group yet. It seems that the main strategy remains as supportive management, including rehydration therapy, electrolyte replacement, and antimotility agents until better treatment options emerge. With the completion of the *C. parvum* genome sequence, new understandings of the biochemistry of mechanisms of

resistance in this parasite have been revealed (Striepen et al. 2004; Umejiego et al. 2004) and more effective drugs against cryptosporidiosis may be available in the future, that are hopefully more widely applicable to all patient groups and affordable for the general public.

Treatment of giardiasis in humans

Metronidazole and albendazole are the drugs most commonly used, either alone or in combination. In one study no difference was found in efficacy of metronidazole versus albendazole, although side-effects as headache, anorexia and abdominal pain occurred more frequently in the metronidazole-treatment group (Karabay et al. 2004). These findings have been supported in later studies, showing that albendazole was equally effective as metronidazole, but with fewer side effects (Solaymani-Mohammadi & Singer 2011).

Single dose treatment using nitroimidazole-based drugs with long half-lives, i.e. tinidazole, secnidazole and ornidazole have also proven to be effective (Escobedo et al. 2014).

Animal studies have shown that metronidazole has genotoxic, carcinogenic and teratogenic potential (Palermo et al. 2004; Tiboni et al. 2008). Reports on possible malformations due to metronidazole treatment are rare (Cantu & Garcia-Cruz 1982), but pregnant women are not recommended to take metronidazole during the first trimester. In such cases, parmomycin has proven to be a safe and effective alternative (Kreutner et al. 1981).

Treatment of giardiasis and cryptosporidiosis in ruminants

There is currently no licensed drug to treat giardiasis or cryptosporidiosis in ruminants, although the need to treat has been questionable. Treatment alone is not sufficient for controlling *Giardia* infection in ruminants because re-infection occurs rapidly and, given the high level of environmental contamination, daily administration of drugs would be needed. Halofuginone is reported to markedly reduce *Cryptosporidium* oocyst output in experimentally infected lambs and naturally and experimentally infected calves; therapy was also reported to prevent diarrhea, and this could be an important consideration as treatment could reduce the extent of environmental contamination and thus onward transmission of infection. Paromomycin sulfate has proven successful in preventing natural disease in a controlled clinical field trial in goat kids, but is not licenced for treatment of this infection per today.

Affected ruminants should be supported with fluids and electrolytes, both orally and parenterally, as necessary until recovery occurs. Cows' whole milk should be given in small quantities several times daily (to the full level of requirement) to optimize digestion and to minimize weight loss. Several days of intensive care and feeding may be required before recovery is apparent. Parenteral nutrition may be considered for valuable calves (O'Handley & Olson 2006a).

Treatment of giardiasis and cryptosporidiosis in dogs

The options for treating dogs against giardiasis are scarce. Two of the most commonly used drug groups are benzimidazoles and nitroimidazoles. The first line treatment is fenbendazole (Scorza & Tangtrongsup 2010), but in cases where giardiasis persists, a combination of fenbendazole and metronidazole can be used. A combination of pyrantel/praziquantel/febantel has shown variable effectiveness in clearing *Giardia* infections (Barr et al. 1998; Payne et al. 2002). Albendazole may induce bone marrow suppression in dogs and cats, and is not recommended for use in these species (Stokol et al. 1997). Quinacrine and tinidazole could be alternative options, but are advised to use with caution due to scarce data on their adverse effects (Scorza & Tangtrongsup 2010).

Before treating dogs many factors of each individual case should be considered: symptoms, contact with other dogs, contact with immunosuppressed people and whether the dog is performing stressful and demanding work, e.g., sled dogs or police dogs (Tysnes et al. 2014).

As no current drug is documented to eliminate *Cryptosporidium* infection in canines, the main goal is to stop diarrhoea by supportive care, such as highly digestible diets used for small bowel diarrhoea which contains fibre and probiotics, and oral rehydration solutions containing glutamine to replace lost absorptive cell surface. Severe dehydration should be treated with parenteral fluid replacement, and in some cases antibiotics to control secondary bacterial infections may be necessary. There are scant publications regarding cryptosporidiosis therapy in cats and dogs, so treatment regimes should be adjusted according to the need of each patient (Scorza & Tangtrongsup 2010).

1.4 *Giardia* and *Cryptosporidium* as waterborne pathogens

Cryptosporidium was discovered to infect humans as late as in 1976 (Nime et al. 1976), and waterborne transmission was confirmed for the first time in 1984 (WHO 2004). *Giardia* was recognized to cause disease in the 1970s (Kreier 1978; Walzer et al. 1971), and waterborne outbreaks of giardiasis has been reported for the last 40 years.

Cryptosporidium concentrations have been reported to be as high as 14,000 oocysts per litre in raw sewage, and 5 800 per litre in surface water, and for *Giardia* the equivalent numbers are 88000 cysts per litre in raw sewage and 240 cysts per litre in surface water (WHO 2004). This magnitude of (oo)cysts in different surface waters, together with the low infectious dose and robust nature, provides the opportunity for large outbreaks to occur when water sources used for drinking water, irrigation, bathing, or other human activities, are contaminated.

The total number of reported waterborne outbreaks has increased dramatically over the last few years, from a total of 325 from the start of the previous century till 2004 (Karanis & Kourenti 2004), to 199 between 2004 and 2011 (Baldursson & Karanis 2011), to 381 outbreaks reported from 2011 to 2016 (Efstratiou et al. 2017). Whether this reflects a real increase in outbreaks, or just greater awareness and knowledge is unknown, but little of such increased knowledge or awareness seems to drip off onto developing countries, as outbreaks from these countries have not been recorded in the last review of worldwide outbreaks, although the authors of the review do note that the true magnitude of waterborne protozoan illness in the regions most affected are still neglected and poorly described, and states a deficiency in the knowledge of frequency and extent of undiagnosed outbreaks worldwide. Establishment of surveillance systems in developing countries is a first step in combating parasitic protozoans and thus improving the health of the population (Efstratiou et al. 2017).

The cost of waterborne outbreaks has been estimated to be high in developing countries. There are direct costs, which include the expenses for prevention or health, e.g. the resources used for medical treatment, and there are direct non-medical costs, e.g. the transportation costs to visit the physician. In addition, there are also indirect costs, e.g., earning losses due to sick leave from work or reduced productivity at work, or resources

spent to provide care for an ill individual, and, in some cases, costs of premature mortality (EPA 2007).

The impact of cryptosporidiosis and giardiasis outbreaks is relatively high due to the large numbers of people that may be involved, as well as the associated socioeconomic implications. For instance, the cost of illness associated with the 1993 outbreak in Milwaukee, USA, where about 400 000 people were infected, has been estimated at US $ 96.2 million (WHO 2004). The outbreak of giardiasis in Bergen, Norway in 2004, where 2500 – 6000 persons were infected had an estimated cost of US $ 5.6 million, and the cryptosporidiosis outbreak in Galway, Ireland in 2007 with 5000 persons infected had a price tag of US $ 5.3 million (Lindberg et al. 2011). These costs average out to about US $ 750 per patient, and does not include additional costs like lost workforce due to sick leave, overtime costs for doctors and technicians, and follow-up of patients suffering from long-term effects of infections. It is worth considering that US $ 750 is most of the monthly wage of a medical doctor in India, and is an amount per patient that is just not feasible to spend on a single patient in developing countries, where occurrences and outbreaks of waterborne protozoans seems to go unnoticed. Nonetheless, these costs and burdens to society are still real for those exposed, regardless of whether it gets attention in media, publications or research projects. There is a need for developing reliable and affordable diagnostic tools for detection and characterisation, as source tracking and prevention are key to preventing or, at least, restricting the spread and outbreaks of waterborne parasites.

1.4.1 Waterborne *Cryptosporidium* and *Giardia* outbreaks and zoonotic implications

Waterborne *Cryptosporidium* outbreaks and outbreak investigations

Some reviews have suggested that calves are the only major reservoir of *C. parvum* infections in humans (Xiao & Feng 2008), and zoonotic sources have been suspected in many water-borne outbreaks. The greatest documented waterborne outbreak in Milwaukee, USA never had its source properly identified, but cattle were initially the prime suspects. Reports have argued that the source may in fact have been human waste drawn from a nearby lake (Zhou et al. 2003). In contrast, the *Cryptosporidium* outbreak in Östersund, Sweden in 2010 was rapidly detected and molecular analysis confirmed the contamination to be of human origin, namely *C. hominis* (Widerström et al. 2014). The tools for tracking waterborne outbreaks have evolved greatly since the Milwaukee outbreak in 1993, and give great advantages in source tracking. Subgenotyping allows us to investigate the sources of contamination more precisely. A study from Thailand found that in three canals receiving municipal, agricultural and industrial wastewater, there was a significant load of *C. hominis*, pointing to a human source of contamination (Diallo et al. 2008). Three drinking-water associated outbreaks occurring over a one-year period in the same area of Northern Ireland was found to be unrelated by utilizing sub-genotyping tools. One of the three outbreaks was associated with a bovine type of *C. parvum*, while the other two were caused by *C. parvum* human genotypes (Glaberman et al. 2002).

The lack of utilizing these tools at the right time may also cause consequences for society and economy, when assumptions of the contaminating source is made. A small waterborne outbreak involving 24 people was reported in 1998 where sheep were suspected of being the source, but molecular studies never confirmed this. An outbreak in England in 1999, where over 200 persons were affected, was strongly associated with sheep grazing around the reservoir, though this was never fully confirmed by molecular analysis (Cotruvo et al. 2004; Qamruddin et al. 2002), and this was also the case in an outbreak of waterborne cryptosporidiosis in Scotland in 2000 (Cotruvo et al., 2004) where *Cryptosporidium* in the faeces of the implicated sheep were not investigated by molecular methods until 3 months after the outbreak, at which time they proved to have *Cryptosporidium* of a different genotype to that of the outbreak (Chalmers et al. 2002). The 1999 outbreak caused several

preventive measures, such as upgrading water treatment plants and removal of grazing sheep to other areas. These implementations led to frustration among sheep farmers (BBC 2000), and particularly so as there was no clear associated disease, and the decision seemed to lack scientific grounding. This led to increased expenses and may as well have caused unjustified stigma around sheep as potential transmitters of disease.

Waterborne *Giardia* outbreaks and outbreak investigations

Aquatic wildlife has been implicated in waterborne outbreaks of *Giardia*. Studies have shown that beavers and muskrats shedding *Giardia* cysts harbour Assemblage B, though there is little evidence supporting these animals are sources of contamination. It has been suggested that these animals are more likely to become infected from water contaminated with faecal material of human or even domestic animal origin, thus acting as amplifiers of the isolate rather than the original source (Fayer et al. 2006; Sulaiman et al. 2003; Thompson 2004). Dogs have also been suggested as being the source of contamination of the water during the waterborne giardiasis outbreak in Bergen, Norway, in 2004, which resulted in thousands of individuals being infected (Landvik 2015), However, the overwhelming evidence was to the contrary, and the genotype associated with this outbreak (Assemblage B) is very uncommon among dogs in Norway (Robertson et al. 2015b). This further underlines the importance of molecular characterization of pathogens in geographical areas so that foci of infection are recognizable and measurements of prevention may be less costly, but more targeted and specific.

Source tracking seems to be a conundrum for many water-borne outbreaks. Most of the 325 outbreaks compiled by Karanis *et al* (2007) did not use molecular methods to verify the genotype implied, leaving the zoonotic implications of the outbreaks unanswered.

1.4.2 Water supply in India and Chandigarh

Although India is one of the countries receiving most precipitation globally, it is in the midst of a serious water problem. Droughts and water shortages are common, but, at the same time, during the monsoon season, there is often too much water and the infrastructure to deal with this is lacking. Whilst the widely reported current flooding due to Hurricane Harvey in Texas and Louisiana USA have resulted in at least 47 deaths, and about 43,000 people requiring emergency housing in shelters, the almost simultaneous flooding due to the monsoons in floods across a swathe of South Asia, including India, have resulted in more than 1,200 deaths, of which at least 500 were in the northern Indian state of Bihar, and with millions of people estimated to have been displaced and at least 700,000 homes destroyed.

Even in non-emergency situations in most cities in India, the operation, maintenance and quality monitoring of drinking water supply systems are inadequate. Water supply pipes leading to individual households in India often have leaking joints, leading to the potential for contamination of the water. In rural areas, even rudimentary facilities of water quality monitoring and surveillance are not available. Similar to most cities in India, the public water supply in Chandigarh is managed by a municipal body, and when the city was built in the 1950s, it was planned that every sector should have access to water for the public by having one or more public water sources, from which the public can either drink directly or collect water to take home, but the quality system of monitoring and surveillance for drinking water quality is by far from optimal (Goel et al. 2015).

1.5 Fresh produce as vehicles of infection for *Giardia* and *Cryptosporidium*

Fresh produce may become contaminated with protozoa and, if eaten raw, may act as a vehicle of infection. Food contamination may occur during production, processing, or preparation.

The biology of *Cryptosporidium* and *Giardia* makes them suitable for transmission via fresh produce; they have a low infectious dose, a robust transmission stage, are small sized, and some genotypes have a zoonotic potential, giving the opportunity for contamination to occur from both animal and human sources (Robertson & Lim 2011; Robertson 2013).

Cryptosporidium and *Giardia* can contaminate food as a surface contaminant. Contamination of foods with small numbers of infectious (oo)cysts in produce that receives minimal washing or treatment prior to ingestion, poses a threat to public health.

As fewer people are likely to be affected in a foodborne outbreak than in a waterborne outbreak, and may be more scattered geographically, a lack of prompt diagnosis may hamper epidemiological investigation. It is worth noting that the last major waterborne outbreak of giardiasis in Europe, in which over 1500 people were infected, took several weeks to be recognised as a waterborne outbreak (Robertson et al. 2006), and, for example, in two documented foodborne outbreaks of cryptosporidiosis, the specific contaminated produce was never detected (McKerr et al. 2015; Pönka et al. 2009).

Contamination of food with protozoa has been considered to occur either directly from food-handlers, perhaps infected themselves or in close contact with an infected person or animal, or from contact with a contaminated environment. Such environmental contamination can be from soil, particularly soil amended with faeces or manure, or from water such as irrigation water or wash water along the food chain (Cook and Lim, 2012; Robertson & Chalmers 2013).

As with waterborne cryptosporidiosis and giardiasis, the potential for foodborne transmission is considered rather similar for both parasites. Indeed, one recent estimate regarding the burden of foodborne disease (Hald et al., 2016), concluded that *Cryptosporidium* and *Giardia* were quite similar regarding source attribution. Kirk et al*.,* (2015) used expert elicitation to estimate that whereas around 15 % of giardiasis cases were

foodborne (uncertainty intervals (UI of 0.08-0.27), a slightly lower proportion (13 %) of cryptosporidiosis cases were foodborne (UI of 0.07-0.24).

Cryptosporidium oocysts and *Giardia* cysts are quite robust, and studies have suggested they can survive numerous treatment processes (Duhain et al. 2012), and remain infective for weeks on stored produce (Macarisin et al. 2010b). Thus, their biology makes them well suited for transmission by fresh produce, as in addition to the infective stages (cysts and oocysts) being robust, the infectious dose is low and infected individuals shed vast amounts of immediately infectious (oo)cysts. Furthermore, the zoonotic potential from both parasites means that contamination of fresh produce may be from infections in humans or in animals. In addition, washing procedures may fail to remove *Cryptosporidium* oocysts as they seem to adhere to surfaces and may infiltrate the stomatal openings of leafy vegetables (Macarisin et al. 2010a; Macarisin et al. 2010b).

In the few documented outbreaks of foodborne transmission of giardiasis, the food handler has been the suspected source of contamination in five of nine cases (Cook & Y.A.L. 2012). However, in the work done as part of this thesis, *Giardia* was found in relatively low numbers on the produce, which may suggest that contamination is more probable from water or another vehicle rather than direct contact with an infected person.

In contrast, among the relatively large numbers of confirmed outbreaks of foodborne cryptosporidiosis, direct associations with food handlers are less common (Robertson & Fayer 2012). However, in some of the work conducted as part of this thesis, I found that some produce was contaminated with high numbers of oocysts, suggesting direct contact with a highly contaminated or infected source, possibly the salesperson or customer.

Foodborne outbreaks transmitted by fresh produce are also difficult to investigate, as by the time an outbreak is identified, the implicated food is usually not available, being either discarded or consumed. Also, as the incubation period for *Cryptosporidium* and *Giardia* may be days, even weeks, so outbreak investigations generally rely on clinical samples and interviews, and environmental inspections.

Many of the foodborne outbreaks for which fresh produce has been identified as the probable vehicle of infection have occurred in Nordic countries, and thus it could be speculated that the climate in these areas may prolong survival of the parasite. However, in

some of the cases the produce implied was imported, and for others the contamination occurred at the serving location.

Indeed, considering the various factors, the association of outbreaks with Nordic countries may be due to the more developed surveillance and outbreak investigations in these regions, as it is not due to higher consumption of fresh produce in these countries (Robertson & Chalmers 2013). In developing countries, where foodborne infections or outbreaks may occur against a background of high infection rates, it is likely that it is difficult to identify when outbreaks occur, and almost impossible to determine the likely vehicles of infection. However, obtaining information on the extent of contamination of different food products with these parasites may provide some pointers regarding the potential for different transmission routes and vehicles for transmission.

Although molecular methods have been used for detection of *Cryptosporidium* oocysts and *Giardia* cysts on fresh produce (Caradonna et al. 2017; Dixon et al. 2013; Rzeżutka et al. 2010), the standard ISO Method relies on isolating (oo)cysts from the sample, and has roots in the standard methods for analysing water for these parasites (ISO 2006; U.S.-EPA 2012), being based on concentration and isolation from debris by immunomagnetic separation (IMS) and detection by immunofluorescent microscopy (Robertson & Chalmers 2013).

These methods for analysing fresh produce for contamination with these protozoa are quite expensive and, as with the standard methods for detection of protozoans in drinking water, may be cumbersome and their value for use in areas where resources are already constrained may be considered questionable.

Another aspect when fresh produce is considered as a potential vehicle for transmission of parasites or other pathogens, is the farm-to-fork chain in the particular situation. In developed countries, where we have availability of all kinds of fresh produce, regardless of season, the chain may be complicated, but it is usually well documented. For example, when contamination of imported strawberries with *Giardia* cysts was identified in Norway in 2016 (Mattilsynet 2017), it was rapidly determined that the strawberries had been imported via a Dutch firm from a particular grower in Spain; this enable investigations and other measures to be implemented at the correct place. In contrast, in developing countries, supermarkets and cold chains are still not common and properly implemented, and produce is commonly

not labelled with place of harvest. My studies were based in India, the second largest producer of fruits and vegetables in the world (Sachdeva et al. 2013) and around 70% of the Indian population depend directly or indirectly upon agriculture; the section below gives a brief overview of the vegetable trade in this situation and how this may potentially exacerbate the sale of contaminated fresh produce, and mean that investigations on the sources of any contamination can be complicated.

1.5.1 The fresh produce trade in India; an industry in change bringing potential pitfalls and possibilities

The distribution and sale of most vegetables in India is via the Traditional Retail Model, which includes many participants. The first links in the chain are the farmers; farms in India are most usually small, with relatively small yield volumes of crop. Farming in India is a low prestige work (Robertson et al. 2015a) and farmers are often economically insecure and struggling financially. Indeed, it is worth noting that farmer suicides account for 11.2% of all suicides in India (N.C.R.B 2014), but it should also be noted that farmer suicide rates are elevated in other countries, including industrialized ones. The farmers sell their produce to agents, who distribute them to commercial customers, vendors, and wholesalers that sell further to retailers. Wholesalers usually deal with a particular range of fresh produce, and the retailers sell the produce directly to customers. In this complicated chain, none of participants will take responsibility for cleaning the vegetables, and transport of vegetables is not monitored. Thus, fresh produce can be transported in soiled containers or in lorries that have, for example, previously been used for transporting animals. The mandis (Indian traditional market places) in Chandigarh are arranged by an alternative system, the Apni Mandi, which was introduced to shorten the chain of intermediaries and increase the income of the farmer and allows only registered farmers to participate and thus sell their produce directly to consumers. This scheme has proven to be financially beneficial for small farmers, although they have the extra burden of organizing the transport and logistics themselves. Problems that have arisen in this system include shortage of storage space, the long duration of market hours, and inadequate facilities such as lighting, drinking water, sanitation, and parking (Ghuman 2000). The mandis are crowded, and many farmers or retailers display their produce on mats on the ground or on slightly elevated stalls. In order

to keep the vegetables looking fresh, the vendors keep a bucket of water, obtained from the most available water supply at the mandi, for sprinkling over their wares.

Figure 6. Traditional mandi and street vendor in Chandigarh, India.

In addition, vegetables may also be sold directly from a vendor with a simple pushcart, who walks the streets with his wares, coming directly to the customers' doorsteps. In the work done in this thesis, I found that vegetables bought from such vendors had the lowest occurrence of contamination; this may initially sound counter-intuitive, however a vendor or retailer that is dependent on a small base of regular customers will do their best to ensure that their customers are pleased with their purchases. Selling a batch of goods that is discouraging to both the eye and the stomach may result in the customers avoiding certain goods or even the vendor or retailer himself.

Although the market and street vendor are established and traditional processes of sale, across the developing world, the retail sector is undergoing fundamental transformation. A large number of domestic and foreign organized retailers (e.g. Big Bazaar, Reliance, etc.) are expanding their business footholds in India (Kolady et al, 2008).

The value-chain model is a new concept for trading of fresh produce in India. Its blueprint is taken from more western retailers, buying directly from farmers and then selling to

customers, avoiding intermediaries completely. The outlay of this system is that fresh produce is cleaned at the arrival hub, and every hub has a warehouse and space available for temperature-controlled storage. However, implementing this production chain in the Indian situation could pose some difficulties, not only due to space and infrastructure limitations may affect both the room for storage and the reliability of the temperature control, but also because Indians often prefer to buy their groceries at their own particular mandi rather than from a shop (Economist 2014). Although there are fewer hands touching the produce in the farm-to-shop chain, this model introduces a further source of contamination as customers in supermarkets in the value-chain model are allowed to make their own choices, rather than pointing out produce that is handed to them by the seller. Customers having the opportunity to pick up and put down items numerous times, means that the fingerprints of contamination can become even more numerous and difficult to trace.

Although the traditional retail model probably remains the prevailing one for sale and purchase of fresh produce in today's India, the western style marketing is gaining a foothold in many Indian cities. Furthermore, the growth of double-income families, who are more likely to prefer convenience food, may result in a significant increase in the size of domestic food markets (Pingali & Khwaja 2004), and probably especially so in relatively developed cities like Chandigarh, where families with reasonably high income tend to settle.

Although it might be assumed that in a country where the dominant religion promotes vegetarianism, the inhabitants would consume more fresh produce than the global average, this is not the case for India, where results of surveys have indicated a consistently low consumption of fresh produce, and 74% of the adult population consumes less than the minimum recommended servings (Hall et al. 2009). However, although it is not common or traditional to consume raw vegetables, as western habits and food outlets are influencing the Indian palate, lettuce and other raw foodstuffs are being consumed more frequently (Vashishtha 2014). If the safety and microbiological quality of fresh produce does not keep pace with the consumption trends, then it can be expected that this may become an increasingly common route for ingesting pathogens, including parasites. A study from Ghana, where fresh salad is not a part of the traditional diet, found that increased consumption of fresh salads through urban fast food inspired from western dishes increased the chance of acquiring parasitic infections as the produce was contaminated due to poor sanitary

conditions during farming and irrigation (Amoah et al. 2007).

Given these rather comprehensive changes for a large market in an enormous country, food safety standards cannot be ignored. A new model for retail and new production chains should also be accompanied by new regulations and routines. Although the government of India has, through the National Horticulture Mission, taken up many initiatives that include handling systems, cold storage, long distance mobile units, pack houses and accreditation of quality testing labs, the implementation of these initiatives is slow and unhurried, and poorly monitored. Gaps may be issues like poor roads, transportation, inadequate electricity, climate variability, inflation, corruption, and political and governance issues (Sachdeva et al. 2013). If food safety is to be recognized as adequate for the consumer, then these issues must be addressed before the corporate models, with mass supplies and large supermarkets, take over the traditional roles of the traditional vendor or small shopkeeper, who has an eye on almost every item in stock.

1.6 Domestic animals as potential sources of environmental contamination with *Giardia* and *Cryptosporidium* in the Indian context

India is home to every sixth person in the world, around 20 million dogs, 200 million cows and buffaloes and 150 million goats (Paul et al. 2014; Singh et al. 2013). Most Indians are Hindus, and even though the Hindu religion speaks of loving and caring for all animals, sometimes the same religion comes in the way of practical animal welfare and disease management. All animal species are generally thought of as being equally worth in Hinduism, but in practice a difference seems to be made. This has a root in cultural, religious, medical and financial factors. The common western view is that there is a difference in between life and quality of life, which might not be as obvious to a Hindu, as pain and misery might be considered as a way to cleanse bad karma. Taking a life means karmic effects of the next life for a Hindu, and issues as letting animals roam freely without food or care, or withholding euthanasia with the justification "we`ll leave it to God" is the righteous way of practice in this culture, who believes that "we are not our bodies, they are merely clothing for our soul"(Prabhupāda 1986), a concept which is hard to fathom for the western world.

1.6.1 Environmental contamination with *Giardia* and *Cryptosporidium* in developed and developing countries

It is clear that in developed countries, the human burden with intestinal parasites is much lower than in developing countries; for example, whereas nearly 2% of adults and 6-8% of children have *Giardia* infections in developed countries, in the developing world one third of the population have had giardiasis (Flanagan 1992). The relatively high prevalence of human infections with *Giardia* and *Cryptosporidium* in developing countries has been attributed to factors as multiple exposure routes due to living and sanitation conditions, cycling through domestic animals, contamination of aquatic ecosystems by the high environmental burden and the robust nature of (oo)cysts against common water disinfectants (Slifko et al. 2000), and, in a vicious circle, the greater number of human infections, coupled with inadequate sanitation and hygiene, result in greater environmental contamination, including water

sources and food, resulting in more infections. Numerous studies have investigated relative prevalences of these parasites in people in different settings, but the role that animals play in environmental contamination has been less explored. In the context of infections with *Cryptosporidium* and *Giardia* in domestic animals, and their contribution to environmental contamination, it is important that not only the cold data of prevalence and infection intensity are explored, but that also the context of animal husbandry within the situation that may also contribute to enhanced exposure routes to the human population.

In the following sections, I describe *Cryptosporidium* and *Giardia* in three important animal groups, goats, cattle and buffalo, and dogs, with particular emphasis on the Indian context and how these animals interact with, and are perceived by, the Indian population. I finish this section with some considerations of animals in Hinduism, as this can also be of relevance regarding animals in parasite transmission cycles.

1.6.2 *Cryptosporidium* and *Giardia* in goats – and goat husbandry in India

In developing countries across the globe, goats make a vital contribution towards cutting the cost of living for many hard-pressed families, and goat populations are increasing. Asia and Africa have experienced the main portion of the total increase in goat numbers, accounting for 89% of the increase between 1979 and 1991 (Mackenzie 2011). and the largest goat population in the world is found in India where it constitutes around 154 million animals (Paul et al. 2014), or around one goat for every 8 people.

The main advantages of goats as livestock are that they do not need expensive housing and feed, and they have impressive abilities in converting limited resources into meat and milk. These factors have boosted goat farming in rural areas and among small-scale farmers in India, as well as their high capacity for adapting to extreme climatic conditions; they are particularly important in arid and semi-arid regions where rainfall may be scant and hence income from crops is uncertain and low (IndiaAgroNet 2017). They have a small body size and docile nature, are prolific breeders, and they thrive better in dry areas, and they may be 2.5 times more economical than sheep under semi-arid conditions. Both female and male animals are of equal value (Sirohi & Chauhan 2011). Furthermore, in India, where beef is rarely consumed, goat meat makes up 35% of the total meat market, as goats are not

associated with any religious taboos among non-vegetarian Hindus, Sikhs, and Muslims in India.

In India, goats are usually not penned in a shed, but rather tethered in the home premises and sometimes, depending on the area, taken for grazing. They are generally reared under semi-scavenging systems. Nearly all goats graze at liberty during daylight hours, and may also be fed kitchen waste, e.g. rice and vegetables, but feed concentrates are usually not utilized. The mortality rate among goat kids (up to 3 months) in India can be as high as 25%, and this is usually attributed to low birth weight and poor milk yield of the ewe (Sirohi & Chauhan 2011).

This means that goat keeping in India is not managed intensively and the goats are viewed as a subsidiary income; the goats are allowed to roam relatively freely and do not receive the routine health care that may be expected in animal husbandry from a western viewpoint.

In western countries, most studies have shown that sheep and goats do not harbour major proportions of zoonotic genotypes of *Giardia* and *Cryptosporidium*, though the management of small ruminants differs greatly between developed and developing countries.

Reported prevalences for both *Giardia* and *Cryptosporidium* in goats around the globe tend to vary considerably. Studies from Belgium (Geurden et al. 2008b), Netherlands (Van der Giessen et al. 2006), Spain (Castro-Hermida et al. 2007; Ruiz et al. 2008) Iran (Jafari et al. 2014) and Uganda (Johnston et al. 2010)have revealed prevalences of *Giardia* in goats ranging between from 12.3% to 42.2%, with the lowest prevalence found in Uganda.

For *Cryptosporidium*, the prevalences in different studies from Belgium, Brazil, Sri Lanka, France and Spain were in ranges varying from 4.8% to 33.6% (Bomfim et al. 2005; Delafosse et al. 2006; Geurden et al. 2008b; Noordeen et al. 2000)

These data might not only reflect the prevalence of infection, but could also be due to the sensitivity of the diagnostic tests used, the age of the goat, and whether only a single or consecutive sample(s) were examined, given the intermittent shedding of *Giardia* cysts, and the acute nature of cryptosporidiosis.

The role of small ruminants in transmission of zoonotic intestinal protozoans in developing countries is not yet defined, and there is a lack of studies defining their part as disease

transmittors or recievers. Given the vast difference of livestock keeping between developed and developing countries, studies made from intensive farms where contact between humans and animals are restricted as well as sanitation and hygiene measures are mandatory by law cannot be compared to the backyard livestock, often housed together with their owners.

In western countries, most studies have shown that sheep and goats do not harbour major proportions of zoonotic genotypes of *Giardia* and *Cryptosporidium*. However, as described above, the management of small ruminants differs greatly between developed and developing countries, and it is possible that in goat husbandry systems as described for India, where the goats are not grazing in designated areas and are living in close contact with their owner, a different situation may occur.

In general, the role of small ruminants in transmission of zoonotic intestinal protozoans in developing countries is poorly defined, and studies investigating their role in zoonotic cycles as disease transmitters or receivers are lacking. Given the vast difference in livestock keeping between developed and developing countries, studies from intensive farms in developed countries where contact between humans and animals is restricted and sanitation and hygiene measures are mandatory are not comparable to the backyard livestock situation seen in countries such as India, where the goats are often housed together with their owners. Thus, the role of goats in the Indian situation, and, in particular investigation of the genotypes or Assemblages of *Cryptosporidium* and *Giardia* found in goats in and around Chandigarh, seemed very relevant in the context of this study.

1.6.3 *Cryptosporidium* and *Giardia* in bovids – and cattle and buffaloes in the Indian context

India today is home to about 200 million cattle, representing one-quarter of the cattle population in the world (Jacobson & Grinker 1999). India is also the largest milk producer in the world (Sudarshan et al. 2007), and most rural families in India own at least one dairy cow (Agoramoorthy & Hsu 2012). However, India has long been unique in possessing so many cattle but without making a maximal profit from cattle slaughter (Harris et al. 1966)

Most cattle in India belong to the species *Bos indicus*, commonly known as zebu cattle. This species, which is extremely hardy and resistant to disease (Jacobson & Grinker 1999), is

central to the agrarian economy of India (Chigateri 2008). Cow dung generates biogas as well as a sustainable source of domestic cooking fuel (Agoramoorthy & Hsu 2012), and they are also used as draught animals in the rural areas (Jacobson & Grinker 1999). India have poor access to fossil fuels, and is therefore more dependent on draught animal power (Ramaswamy 1998).

In addition to cattle, the domestic water buffalo (*Bubalus bubalis*) is a species of paramount importance to India; as well as being a major source of milk and meat (for those who eats it), it is also a considerable supplier of draught power. Water buffalo are usually classified into two major categories, the swamp and river buffalo. The river type is the one found on the Indian subcontinent (Kumar et al. 2007).

Figure 7. River buffaloes in a peri-urban area of Chandigarh

In the Hindu religion, Gods and Goddesses are sometimes incarnate animal forms. The cow is a microcosm of the universe, and, to a Hindu, the cow is the most sacred of all animals

(Agoramoorthy & Hsu 2012; Stevenson 1954). Contact with cow products, such as milk, ghee, urine, and dung is considered purifying, and many Indians have a custom of applying dung-paste on the floor daily for religious purification and protection (Jacobson & Grinker 1999; Korom 2000). In addition to the cow being sacred in Hinduism, it is also venerated in Sikhism, Jainism, and Buddhism, all widespread religions in India. Due to this sacred status, India has banned cow slaughter by law in all states except two (Agoramoorthy & Hsu 2012), and in some states it is not even allowed to kill infirm or dying cows (Chigateri 2008). In the states bordering Chandigarh, Haryana and Punjab, slaughter of cows is totally prohibited. In Punjab, anyone violating the law can be punished with imprisonment up to a maximum of 2 years whereas in Haryana the rule is even stricter, with violation of the law punishable by rigorous imprisonment up to 10 years; in both states the burden of proof is placed upon the accused, the offence is non-bailable and cognizable, meaning that an arrest can be made without a warrant and thus fall into the category of crimes such as crimes like rape, murder, and theft rather than crimes like public nuisance. Indeed, mob violence against those suspected of slaughtering cows or eating beef has risen, with several associated brutal beatings, some resulting in death, by self-appointed vigilante “cow protectors” during the last couple of years.

Figure 8. Urban cattle catchment, and a cattle farm in the peri-urban area of Chandigarh

Although worshipped and holy, cattle seldom die of old age in India, as they are starved or neglected until they die a “natural” death. Stray cattle are usually older cattle that have reached the end of their productive years and are left out onto the streets. Cow sanctuaries, or *Gaushalas,* provide shelter for hundreds of these cattle, but do not have sufficient capacity to cope with the whole stray cattle population (Singh et al. 2013). The Indian Veterinary Council has estimated that there is only food to sustain 60% of the Indian cattle population (Kang 2003), and the rest are left to starve or roam the streets in search for food, where garbage makes up the bulk of the diet. In New Delhi alone, there is an estimated 40 000 cows roaming the streets, spreading garbage and constituting traffic hazards (Agoramoorthy & Hsu 2012) .The problem with stray cattle and traffic accidents is also becoming an issue in Chandigarh, where the stray cattle population has risen in recent years, together with traffic incidents (Victor 2013). The increase in stray cattle population is likely due to the rise in urban dairy farming in India, as the growing middle classes in the cities are increasing the demand for dairy products.

This management of husbandry on a countrywide basis is quite unique for India. People in industrialized nations may find it difficult to fathom the extent to which the cow, or cattle in general, is being worshipped and neglected at the same time, in a country that could benefit immensely from effective husbandry. It has been argued that Hindu cattle practices are irrational since religious and political prohibitions on killing and eating cows means wasting animal products, while at the same time sustaining an unproductive cattle population (Jacobson & Grinker 1999).

In addition, this close relationship between cattle and cattle products fades the interface of human-animal interaction. In western countries, applying cow dung to the floor of your house would be considered quite eccentric, while in India this is a respected tradition. Although there is no easy solution regarding segregation of cattle and people, education regarding risk factors and proper hygiene could lessen the pathogen interface between humans and animals. With regards to the work described in this thesis, it was obviously of interest to investigate the role that cattle play in the transmission cycles of *Cryptosporidium* and *Giardia.*

Infections of cattle with *Cryptosporidium* species and *G. duodenalis* assemblages of zoonotic potential have been frequently reported, indicating that cattle are a reservoir for human

cryptosporidiosis and giardiasis (Hunter & Thompson 2005; Ryan & Cacciò 2013). Cattle are commonly infected with *C. parvum, C. andersoni* and *C. ryanae* (Xiao 2010), of which *C. parvum* is of particular importance as a zoonotic species. Contact with infected calves have been identified as a main reason for a number of small cryptosporidiosis outbreaks in veterinary students, research technicians, and children on farm visits, validating the zoonotic potential and transmission of cryptosporidiosis from cattle (Gait et al. 2008; Kiang et al. 2006; Preiser et al. 2003). In addition, case-control studies have reported that contact with cattle is a significant risk factor for sporadic cryptosporidiosis in humans (Hunter et al. 2004; Roy et al. 2004).

In dairy cattle, *C. parvum* is mostly found in pre-weaned calves and *C. andersoni* in yearlings and adult cattle (Santín et al. 2008; Trout et al. 2006) Prevalences have been found to vary from 1% (Kváč et al. 2006) to 59% (Olson et al. 1997) in calf holdings, and up to 100% on farm level (Santín et al. 2004). The highest prevalence has been observed in calves up to 5 weeks of age (Quilez et al. 1996).

In cattle, the livestock specific *Giardia* Assemblage E is considered the most prevalent, though up to 59% zoonotic assemblage A isolates and mixed infection with both E and A have been reported (Geurden et al. 2008a; Sprong, H. et al. 2009). This would suggest that calves may be considered as a potential reservoir for human infection, although within assemblage A sub-assemblage AI is predominantly found in livestock and companion animals, while assemblage AII is more prevalent in humans (Feng & Xiao 2011). Prevalences have been found up to 73% (Olson et al. 1997), and on farm level it can be as high as 100% (Geurden et al. 2010; Geurden et al. 2012; Olson et al. 1997). Nevertheless, the public health risk from bovine giardiasis is generally considered to be minimal, as it has been estimated that about 80% of *Giardia* infections in dairy cattle and 98% in beef cattle are due to assemblage E (O'Handley & Olson 2006b). Studies from developed countries has shown that less than 20% of cattle in a herd may harbour the most common zoonotic genotype, Assemblage A (Santín et al. 2009; Sprong, Hein et al. 2009; Trout et al. 2004; Trout et al. 2005), Longitudinal studies have indicated that infections of zoonotic genotypes of *Giardia* in cattle may be transient, as mixed infections of Assemblages A and E have been reported frequently in cattle, and it has been suggested that immunologically mature cattle are able to resist infection with assemblage A, while the host-adapted Assemblage E is capable or

establishing infection (Uehlinger et al. 2011). Yet another longitudinal study found a 43% occurrence of Assemblage A concurrently with 57% Assemblage E in adult cattle (Uehlinger et al. 2006).

However, whether these data from studies predominantly from North America and Europe are indicative of what might be expected in India, the country of the holy cow, is less certain. There are some studies published regarding *Cryptosporidium* prevalence of bovines in India. (Bhat et al. 2012) found an overall prevalence of about 30% in buffalo calves in Punjab, with the highest incidence in calves under one month, and the highest rate of infection during the monsoon season. A study from six different states in Southern India found prevalences ranging from 86.7% to 17.7% between the states. A wide variety of *Cryptosporidium* species were identified, including *C. andersoni, C. ryanae, C. parvum* and *C. bovis,* with *C. ryanae* the major species found, and the zoonotic *C. parvum* only accounted for 4% and only found in one of the six states examined (Venu et al. 2012). A study from six targeted states ranging all over India geographically, from South to North, revealed *Cryptosporidium* prevalences from 5.4% to 30.8%, with the lowest prevalence found in the southern parts, and the highest in the northern parts of the country. In contrast with the previous study, the only species identified in this study was *C. parvum*, and the highest occurrence was found in calves during the monsoon season (Paul et al. 2008). Maurya et al. (2013) also examined the prevalence of *Cryptosporidium* in bovine calves in 3 different climatic regions in India and found a higher prevalence (35.4%) in the northern sub-temperate parts, and the lowest prevalence in sub-tropical plains (13%), with a significantly higher prevalence of infection during the monsoon season. Again, the only species identified in this study was *C. parvum*.

Molecular tools using markers in the 60 kDa glycoprotein (gp60) gene have improved the ability of identifying and differentiating zoonotic *Cryptosporidium* at the genotype and subgenotypic levels. These are useful for investigating the distribution of *C. parvum* variants in cattle and human populations in different geographical regions (Robertson 2014b). Although the majority of publications revealed a strong foothold of *C. parvum* in the cattle and buffalo population of the country, none of the *Cryptosporidium* studies mentioned above utilized this tool, or other subtyping approaches to further assess the zoonotic potential of *Cryptosporidium* shed from bovines in India. Thus, of particular interest for the investigations in this study was to investigate the sub-types, as well as the species, of

Cryptosporidium occurring in the bovid populations in and around Chandigarh, in order to provide some data on the likelihood of spill-over occurring into the human population, as some data on *Cryptosporidium* subtypes in people in Chandigarh have been published (Sharma et al. 2013).

Furthermore, few studies regarding the zoonotic potential of cattle in India have been published, though a study from West Bengal found a 12.2% prevalence of *Giardia* on a dairy cattle farm, where also 27.5% of the farm workers were infected; however, although approximately 12% of the *Giardia* isolates found in humans were of Assemblage A, under 3% of *Giardia* in the cattle were also of Assemblage A (Khan et al. 2010). This seems to be in concordance with the broad findings from studies in developed countries regarding the distribution of genotypes, and for the present study the intention was to investigate whether, in the Indian context, where relevant transmission factors, such as housing conditions and management, differ wildly from those in Europe and North America, may provide similar or different findings. Thus, the question being addressed partly included whether having an apparently much greater opportunity for transmission of *Giardia* between humans and cattle, resulted in any genetic barrier being defeated.

1.6.4 *Cryptosporidium* and *Giardia* in dogs – and man's best friend in the Indian context

The population of dogs worldwide may be as high as half a billion (Hsu et al. 2003), and free roaming dog populations have emerged as both animal welfare and public health problems in developing countries, where they also face welfare issues such as malnutrition, starvation, disease, and abuse. Additional social problems include bites, road accidents, fighting, noise, faecal contamination, uncontrolled breeding and spread of garbage. Approaches to the management of free-roaming dog populations have changed over the past twenty years, from capture-and-kill to mass vaccination, spaying, habitat control, and responsible dog ownership, though cultural differences in views of dog ownership and the role of dogs in society influence the prevalence of dogs, the condition of stray dogs and dog – control policies In India, there has been a rise in the canine population, both due to the shift to an animal birth control programme instead of euthanasia in response to animal welfare activists, and also due to the increase in popularity of keeping dogs as pets in middle-class

urban families. The success of animal birth programmes depends on sterilizing 70% of stray dogs in a given area, and, due to the limited funds and resources allocated to this programme, achieving this target seems an uphill, if not impossible, task (Menezes 2008; Totton et al. 2010; Totton et al. 2011). Dog faeces in open spaces and public areas, left by stray dogs or irresponsible dog owners, could represent an important source of zoonotic pathogens, and among the parasites this may include not only *Cryptosporidium* and *Giardia*, as discussed for cattle and goats, but also various helminths, including *Toxocara canis*, taeniid worms (including *Echinococcus* spp.), and hookworms, such as *Ancylostoma* spp.

Although finding the same pathogen species in both human and animal populations, does not necessarily indicate that zoonotic transmission has taken place, molecular studies may provide good indications on pathogens` emergence. It is still more useful to explore the dynamics of transmission between humans and animals living in the same household or endemic focus. Some studies have investigated the transmission dynamics of *Giardia* between dogs and humans living in close proximity, and a few of them found no significant relation of genotypes shared with dogs and humans (Cooper et al. 2010; Traub et al. 2004), whereas other studies have found a strong correlation between humans, dogs, and *Giardia* genotypes (Inpankaew et al. 2007; Traub et al. 2009). In addition, a study from Assam in India found a dominance of zoonotic assemblages in both humans and dogs living on the same tea estate. This was further backed up by epidemiological data showing a significant association between *Giardia* infection in humans and the presence of a *Giardia* positive dog in the same household. However, it was also noticed that 30% of these dogs also had eggs of *Ascaris lumbrocoides,* a human-specific roundworm, in high intensities in their faeces, indicating coprophagy of human faeces and thus mechanical carriage, which could also have been the explanation for the *Giardia* cysts of the same genotypes to those found in the people (Traub et al. 2003; Traub et al. 2004).

It seems there are no general answers to the zoonotic potential of *Giardia* infection in dogs to humans, and each endemic focus should be considered uniquely with a focus on management, sanitation and human-animal interfaces.

Unlike with cattle, that the Indian population worship and respect, there is a wide religious and cultural distance to the dog in Indian culture, sprinkled with a legitimate fear of roaming dogs, as they are well-known in this country as transmitters of rabies virus through their

bites. In India, a person is bitten by an animal every two seconds, and about 15 million people are bitten every year, mostly by dogs (Menezes 2008; Sudarshan 2005; Sudarshan et al. 2007), so it is not hard to understand why stray dogs are regarded as dangerous and unwanted by the general public. Indeed, if the cow is the most respected animal in the Hindu religion, the stray dog might be the least respected animal. While many parts of the cow are considered pure and purifying, it is quite the opposite for dogs (Prabhupāda 1986). Nevertheless, it has been suggested that there are 20 million stray dogs in India (Traub et al. 2005), and the Indian pet dog population amounts to 28 million animals (Sudarshan et al. 2007), thus despite their lowly status there is clearly a large canid population, and as clearing up dog faeces is not considered a normal reaction in Indian society, there remains the potential for dogs in India to contribute to environmental contamination with potentially zoonotic parasite transmission stages (Traub et al. 2005).

In order to investigate the role of dogs in the parasite transmission cycles in Chandigarh, some of this work investigated the parasites of dog faeces sampled from Chandigarh's green spaces.

1.7 Knowledge gaps

Although many studies have investigated the prevalence of intestinal parasites in human populations in India, and also the impact (morbidity and mortality) that diseases such as giardiasis and cryptosporidiosis have on the paediatric population, we have very little knowledge about the most common transmission routes, risk factors for infection, and the role that animal infections may have in acting as reservoirs of infection.

Furthermore, although waterborne outbreaks of giardiasis and cryptosporidiosis have been reported from developing countries, we have scant information about such outbreaks in developing countries, despite logic indicating that in countries where the infrastructure is poorer should be more vulnerable to those contamination events that are likely to lead to such outbreaks.

This has led to a number of questions:

- What is the extent of contamination of potable water with intestinal parasites in India and what are the risk factors for contamination?

- If contamination occurs, are the species concerned of infection risk to people consuming the water?

- What is the extent of contamination of fresh produce with intestinal parasites in India, which types of fresh produce are most at risk, and are there any obvious risk factors for contamination?

- If contamination occurs, are the species concerned of infection risk to people consuming the fresh produce?

- Which domestic as well as stray animals living in close contact with the human population act as reservoirs of infection with zoonotic intestinal parasites, what are the risk factors for infection in these animals, and is the likelihood of contamination of water or fresh produce with such parasites greater from infected animals or infected humans?

- Do we have appropriate tools to address these questions in a resource-poor setting?

- If contamination of fresh produce with the transmission stages of intestinal parasites occurs, what factors may increase or decrease the likelihood of survival of these transmission stages prior to ingestion by the next susceptible host?

- How does seasonal weather shifts affect both parasite prevalence in different hosts and the likelihood of contamination of potential vehicles of infection?

2. Aims of study

The work described in my thesis was nested within the structure of a larger project called "Intestinal parasites in Northern India: effects of climate change patterns on prevalence of different intestinal parasites in children" (short title: Para-Clim-Chandigarh), funded through the New Indigo Partnership Programme (an EU-India Innovation joint funding programme). This project was intended to be a collaboration between three research facilities; NMBU in Norway, Institut Català de Ciències del Clima (IC3) in Barcelona, Spain, and the Postgraduate Institution of Medical Education and Research (PGIMER) in Chandigarh, India. Within the Para-Clim-Chandigarh project were a total of four work packages, with three of them constituting independent research projects based on the experience and expertise of the three partners, and that were intended to interact and feed into each other in order to achieve the ultimate aim (see figure 9 below).

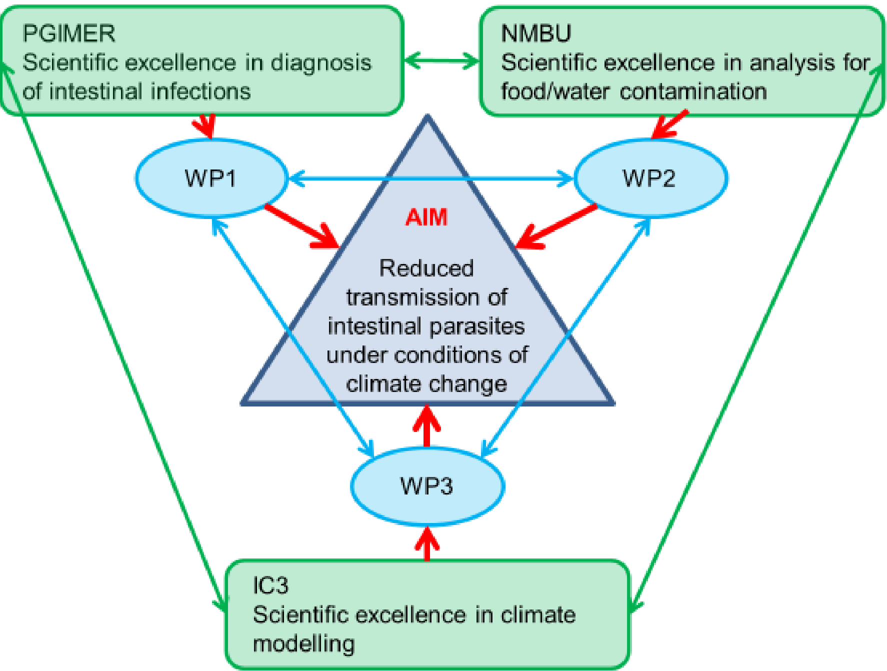


Figure 9. Interaction between the three institutes and the three main work packages in the Para-Clim-Chandigarh project (Picture acknowledgement, Lucy J. Robertson).

The Para-Clim-Chandigarh project thus consisted of short-term and long-term objectives.

The short-term objective was to investigate foodborne, waterborne, and zoonotic transmission of intestinal protozoan parasites in Chandigarh, and the impacts on their transmission of large-scale and regional climate patterns and their interaction with the local environment in the light of projected climate change for the region.

The long-term objective was to develop workable initiatives that could reduce transmission of these pathogens and provide early – warning systems.

As described, in order to achieve these objectives, the project plan was divided into different work packages, WP1, WP2, WP3 and WP4, and each of the partners had particular specialist expertise relating to the assigned work package, giving the project a synergistic approach. Whereas the first three work packages were research based, and the intention was the combined results would be the basis for an initiative to reduce field prevalence or provide a basis for an early warning system.

These basic elements of each of the work packages were (see Figure 10):

1) Assessing the prevalence of intestinal parasites in children in different regions of Chandigarh, with particular focus on *Cryptosporidium* and *Giardia*, including epidemiological, demographic, and clinical considerations. In addition, data from records going back decades will be used to assess temporal changes in prevalence.
2) Investigating the potential for waterborne, foodborne and zoonotic transmission of these parasites due to contamination and the impact of different weather patterns on a seasonal level on the extent of contamination.
3) Assessing the effect of regional climate and the local environment on the prevalence of these parasites in children, with modelling based on over 30 years of data.
4) Dissemination and use of the results through not only academic outputs (PhD theses, conference communications, scientific articles), but also stakeholder workshops and other local initiatives.

Details of WP1, WP3, and WP4 are not described here but can be found in the project documents elsewhere. The work in my thesis is nested within WP2, and this is described in greater detail.

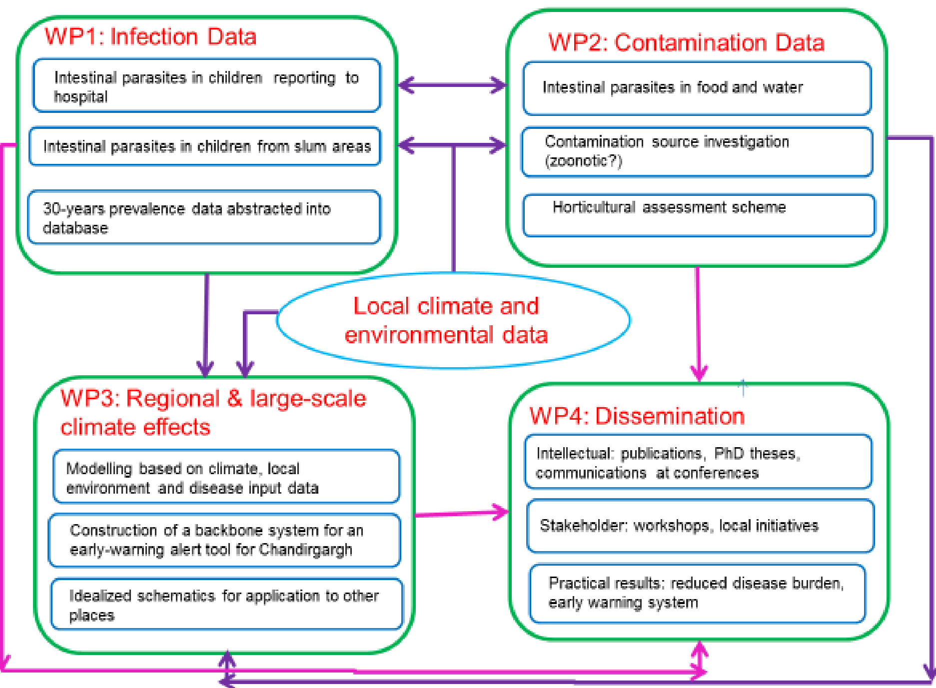


Figure 10. The main elements of the four work packages in the Para-Clim-Chandigarh Project (Picture acknowledgement, Lucy J. Robertson).

WP2: WP2 provides the baseline, funding and all activities regarding this thesis. It has been executed with a base at the Norwegian University of Life Sciences, in the parasitology laboratory, with extended visits to Chandigarh to collect data and samples during different seasons.

The role of NMBU in this project was to assess contamination of water and different fresh produce in and around Chandigarh using methods already developed (or that could be modified for use in this project) and to examine animal populations living in close contact with the human population of Chandigarh for the prevalence of intestinal protozoans and assess the zoonotic potential thereof using molecular methods. The samples were collected during different seasons and over prolonged period to assess any affect of season or local

weather conditions on prevalence. Other factors, such as within city differences, were also to be assessed.

It was intended that the data obtained from the food, water, and animal surveillance would be considered initially in conjunction with the data obtained in WP1, and subsequently in the light of the data in WP3. Due to economic restrictions with the partner (IC3) responsible for WP3, this was run at a reduced rate and the data are still under evaluation. Furthermore, the partner responsible for WP1 were unable to fulfil their contribution to the project.

The objectives for this thesis were thus based on the plan for WP2, and can be considered under the following main objectives:

2.1 Main objectives

1. Develop and/or modify methods to determine the occurrence, prevalence, and concentration of food- and waterborne parasites (with focus on *Cryptosporidium* oocysts and *Giardia* cysts) which can be applied to laboratories or projects on low budgets.

2. Identify the extent to which fresh produce for sale in Chandigarh and drinking water supplied in Chandigarh is contaminated with *Cryptosporidium* and *Giardia*, the species and genotypes of these parasites, and investigate any effect of seasonal or other factors on contamination of fresh produce and drinking water in Chandigarh with these parasites.

3. Identify sources of zoonotic intestinal parasites in Chandigarh, with particular emphasis on *Cryptosporidium* and *Giardia*, and their possible seasonal variations. Identify any risk factors particularly associated with zoonotic parasites identified in animal hosts.

In order to break down these rather large objectives into more manageable tasks the following sub-goals were specified and are addressed in the work described in this thesis:

2.1.1 Sub-goals

1. Modify standard methods for analysis of fresh produce and water for contamination with protozoan parasites with the aim of reducing the cost, such that more samples can be analysed within budget, but without losing method efficiency. Ensure the validity of the methods developed through use of multi-lab testing.

2. Use the methods developed on samples collected from potable water sources and vegetable markets in Chandigarh, and thus estimate the occurrence and species/genotypes *Cryptosporidium* and *Giardia* as contaminants in food and water that may infect humans, as well as any associations with environmental factors such as location and season/weather.

3. Investigate whether contamination of fresh produce by *Cryptosporidium* oocysts or *Giardia* cysts necessarily means that the produce has infectious potential. By using laboratory-based spiking studies of fresh produce, assessing the survival of these parasites over time kept under typical household storage conditions.

4. Identify the domestic animal species that have the potential to act as reservoirs of infection for intestinal parasites (particularly *Cryptosporidium* and *Giardia*), for people, and investigate prevalences and species/genotypes:

a) Goats are a livestock on the rise in developing countries, and provide economic relief for families on restricted budgets. This form of animal husbandry is commonly based on free-ranging goats, often living close to, and sometimes in the same house as their keepers. I wanted to assess whether these families and goats also shared intestinal protozoan parasites as well as households.

b) In India, bovines are roaming freely and are kept and worshipped close to humans, and Chandigarh is no exception. I wanted to assess whether these animals harbour zoonotic intestinal protozoans and the possible seasonal variation in the shedding of them.

c) In Chandigarh, and India in general, stray dogs are a population on the rise. Previous studies have indicated that dogs, whether strays or pets, in resource constrained settings may act as carriers of zoonotic intestinal protozoans and helminths. I wanted to assess whether this was the case for the dogs in Chandigarh, and whether there was any seasonal variation in the shedding of these parasites.

3. Summary of papers

The papers published or prepared in association with this thesis are presented here in two groups, research papers directly addressing the objectives of the project, and review or opinion papers that help to place this project in context.

Research papers

Paper I

A reduced cost-approach for analyzing fresh produce for contamination with *Cryptosporidium* oocysts and/or *Giardia* cysts.

Summary of paper

In this research article, a modified method for analysing fresh produce for contamination with *Cryptosporidium* oocysts and *Giardia* cysts is optimised, and validated via an international ring-trial involving 11 participating laboratories. The work for developing this method, which is based on a recently published international standards (ISO 18744:2016) was begun as part of a previous project, and was refined, tested, and validated as part of my work. Based upon elution and immunomagnetic separation for isolation, and fluorescence microscopy for detection, the important modification involves the use of fewer beads and different buffers to reduce the cost of each analysis by 80% whilst retaining recovery efficiency. This method may be useful for research projects or survey work in which large number of samples need to be analysed, or laboratories investigating contamination events in circumstances where constrained resources do not permit use of the standard ISO method.

Paper II

Keeping it cool: Survival of *Giardia* cysts and *Cryptosporidium* oocysts on lettuce leaves

Summary of paper

Why are there more reported foodborne outbreaks of cryptosporidiosis than giardiasis, as they are both well suited for transmission through fresh produce? The experiments described in this research article aimed to partly answer this question by assessing the survival of *Cryptosporidium* oocysts and *Giardia* cysts stored under different household conditions. *Giardia* cysts were found to be not as robust as *Cryptosporidium* oocysts when kept at ambient temperatures and died more rapidly, but both parasites survived for long periods when refrigerated. This suggests that although fresh produce may be an appropriate transmission vehicle for *Cryptosporidium*, it may be less ideal as a vehicle of transmission of infection for *Giardia*. Thus, unless kept in cool, moist conditions, *Giardia* cysts on fresh produce are unlikely to survive. This provides some explanation as to why most foodborne outbreaks of giardiasis have been associated with food-handler contamination at the point immediately prior to consumption, whereas many foodborne outbreaks of cryptosporidiosis are associated with contamination relatively distant from the site of consumption, even in another country.

Paper III

Checking the detail in retail: Occurrence of *Cryptosporidium* and *Giardia* on vegetables sold across different counters in Chandigarh, India.

Summary of paper

In the research described in this article, vegetables traditionally eaten raw were sampled from different retailers and socioeconomic areas in Chandigarh, India, and analysed for presence of *Cryptosporidium* oocysts and *Giardia* cysts on their surfaces. The results showed an overall prevalence of 5% for *Giardia* contamination and 6% for *Cryptosporidium* contamination, with no obvious association with sampling season or type of produce. These results are in line with those obtained from other similar surveys. Some of the parasites detected were analysed using molecular techniques and the species and genotypes of the

contaminating parasites were ascertained. One unexpected finding was that traditional retailers, the vendors and open markets, were less likely to be associated with contaminated fresh produce than the up-and-coming supermarkets. Furthermore, the intensity of *Cryptosporidium* contamination, in terms of oocysts per gramme of produce, was significantly higher in the supermarkets. This may reflect the mode of sale in supermarkets, where customers are able to handle the produce and make their own selection. In traditional sales, the vendors themselves handle the produce and pass it to their customer.

Paper IV

Is drinking water making waves in Chandigarh? Occurrence of *Cryptosporidium* and *Giardia* in potable water sources.

Summary of paper

An established method of analysis of water for contamination with *Cryptosporidium* and *Giardia* was modified in a similar way to that described in Paper I for fresh produce, to give a method with the same recovery efficiency but at a lower cost. This method was used to analyse 71 potable water samples collected from and around Chandigarh over a period of 18 months; over 20% of the water analysed was found to be contaminated with either *Cryptosporidium* or *Giardia*. Although seasonal variation in contamination was not found, this could reflect the unusual climatic conditions during the period of sampling. One significant finding was that water samples from the more wealthy areas of the city were less likely to be contaminated with protozoan parasites.

Paper V

Goats in the city: prevalence of *Giardia* and *Cryptosporidium* in extensively reared goats in northern India

Summary of paper

Goats are a very popular homestead livestock in India, where they are often kept to supplement an already scarce income. They are often left to graze freely in the surrounding environment and are frequently housed under the same roof as their owners, where hygiene and sanitation standards are often low. This situation is very different from livestock keeping in industrialised countries, and means that the barrier between human and animals with regards to transmission of zoonotic diseases is relatively low. In this article, goats from extensive farms and backyards were sampled, to assess the prevalence and zoonotic potential of *Cryptosporidium* and *Giardia*. A prevalence of 34.3% for *Giardia* and 0.5% for *Cryptosporidium* was found; coupled with the high prevalence of *Giardia* was the finding that the majority of *Giardia* genotypes were potentially zoonotic. This contrasts with the findings of previous studies on *Giardia* in goats, in which most *Giardia* isolates are of the non-zoonotic Assemblage E. These results suggest that *Giardia* may be transmitted regularly between goats and people in their environments, potentially to the detriment of both.

Paper VI

Prevalence and zoonotic potential of intestinal protozoans in bovines in Northern India

Summary of paper

Cattle have a dual position in India, being both worshipped and used in the food industry as providers of dairy products. This paradoxical existence means that they are both protected, but often left to fend for themselves as there are no clear owners of the livestock, and any veterinary or other interventions may be against religious practice and often difficult to apply. As these animals may roam the streets in search for food, often alongside their human counterparts, the potential of zoonotic transmission of parasites may be high. In this paper, the prevalence and zoonotic potential of *Cryptosporidium* and *Giardia* was assessed

in the bovine population in and around Chandigarh. The overall prevalence was 8.2% for *Giardia*, and 2.4% for *Cryptosporidium*, with calves having the highest occurrence. Non-zoonotic assemblages were predominantly found in the case of *Giardia*, and for *Cryptosporidium*, zoonotic subgroups previously described from infected humans in the same area were identified as well as non-zoonotic genotypes.

Paper VII

Not just a walk in the park: prevalence and seasonal variation of parasites in faeces shed in recreational parks in Chandigarh, India.

Summary of paper

The stray dog population is on the rise in India, and packs of animals spend their lives in and roam the same parks as humans. In this research article, the zoonotic potential of parasites in dog faeces left in recreational parks was assessed, as well as their seasonal variation and distribution among social layers of the city. The results showed that dogs and humans in India are not only culturally separated, as the *Giardia* assemblages revealed from faecal samples were mostly canid-specific and the helminth prevalence was low.

Review and Opinion papers

Paper VIII

Climate change and foodborne transmission of parasites: a consideration of possible interactions and impacts for selected parasites.

Summary of paper

In this review article, different foodborne parasites and their biology is reviewed in the light of climate change and the implications that climate change on the foodborne transmission of such parasites is discussed

Paper IX

Keeping parasitology under the One Health umbrella.

Summary of paper

In this opinion article, the importance of parasites as threats to public health are discussed. Although viruses causing acute disease (e.g. Ebola, MERS) may take the attention of the media and research, parasites are just as widespread and may cause debilitating disease. Written just before the major Ebola epidemic, this article takes MERS and Chagas Disease as representatives of important viral and parasitic pathogens and compares them. Although parasitic disease often has a less dramatic, more insidious, effect upon its hosts, there is a clear need for parasitic infections to step into the limelight of attention of researchers and interdisciplinary health personnel such as doctors and veterinarians.

4. Materials and Methods

4.1 Diagnostic tools for environmental samples

4.1.1. Tools for detection of *Cryptosporidium* oocysts and *Giardia* cysts in potable Water

Both *Giardia* and *Cryptosporidium* are able to survive well in the aquatic environment, and *Cryptosporidium* oocysts are resistant to chlorine at the concentrations used for treatment of drinking water, thus the absence of indicator bacteria does not indicate the absence of *Cryptosporidium* oocysts or *Giardia* cysts. The international standard methods are applicable for examination of potable and recreational water, which saw the light of day in the early 1980`s in USA, and have since then been further improved, leading to the most commonly used Standard Methods today which are US EPA Method 1623.1 (2012)and ISO method 15553 (2006).

The procedure consists of three consecutive steps; Concentration (most often by filtration), separation (isolation by immunomagnetic separation – IMS) and detection (by fluorescent microscopy using specific fluorescent labels). All these steps are expensive, and there is currently only one company supplying an IMS kit which is suitable for isolation of both *Giardia* cysts and *Cryptosporidium* oocysts. The fluorescent labels are not expensive, but a fluorescent microscope is, and such analyses should be performed by trained operators. A laboratory performing such analyses should also be established and respected in this field, with quality control data and regular participation in ring-testing. All these documentations and training results in further costs and falls on the provider of the water sample. If a sample is to be found positive, the cost of molecular analysis will be added to the bill (Robertson 2014a).

Concentration

This step is crucial, and its efficiency will largely affect the further steps, as well as the recovery rate (% of initial number of (oo)cysts in the water that can be recovered). The filtration techniques currently applied are filtration, flocculation and continuous flow centrifugation.

Filtration

This technique concentrates (oo)cysts on filters. Cartridge, membrane or ultrafiltration can be applied depending on the type of filter. Difference in the recovery rate between filters have been observed (DiGiorgio et al. 2002; Helmi et al. 2011; Lee et al. 2004; Wohlsen et al. 2004), but also the matrix of the filtrate may hamper the isolation step. It has been shown that factors as nature of solids, turbidity and pH of the sample may affect the filtration step (DiGiorgio et al. 2002; Feng et al. 2003).

Cartridge filtration

This procedure allows for 10 – 1000 litres of water to pass at a flow rate of 1-5 L per minute through filters with a pore size of 1µm. The trapped material is then eluted and concentrated by centrifugation. This type of filtration is listed in the USEPA method 1623 for the concentration of *Cryptosporidium* oocysts and *Giardia* cysts in water.

Membrane filtration

In membrane filtration, large diameter membrane filters of nominal pore size ranging between 1 and 3 µm are applied (Ongerth & Stibbs 1987; Shepherd & Wyn-Jones 1996; Wohlsen et al. 2004). Flatbed membranes are generally prone to earlier clogging than cartridge filters when processing turbid samples and therefore suit better for water with low turbidity and treated water (SCA, 2010). (Oo)cyst loss has been reported and scraping of the filter has been suspected to damage recovered (oo)cysts (Stanfield et al. 2000; Wohlsen et al. 2004).

Ultrafiltration

Ultrafiltration has been shown to be more efficient and robust at recovering *Cryptosporidium* oocysts and *Giardia* cysts from different water matrices. In addition, it is less expensive than cartridge filters. (Francy et al. 2013; Hill et al. 2005; Hill et al. 2007; Hill et al. 2009; Rhodes et al. 2011; Rhodes et al. 2012).This method is nonetheless currently not standardized as an ISO – method.

Flocculation

Flocculation concentrates (oo)cysts with calcium carbonate (Vesey et al. 1993). This technique is not recommended as it has a poor recovery rate compared to filtration (Feng et al. 2011), and it may affect the viability of the (oo)cysts (Campbell et al. 1995).

Continuous flow centrifugation

Portable continuous flow centrifugation (PCFC) has been approved by USEPA method 1623 for concentration of *Cryptosporidium* oocysts and *Giardia* cysts in different types of water samples. The technique is cost-effective and can be used for the simultaneous concentration of pathogens over a wide size range, including bacteria, and is suitable for monitoring large volumes of potable water for contamination with protozoa (Zuckerman & Tzipori 2006).

Immunomagnetic separation

Immunomagnetic separation (IMS) involves the attachment of (oo)cysts with para-magnetic beads coated with specific antibodies, the beads are separated from the unwanted particulate matter using a magnet and then the (oo)cysts are dissociated from the beads using acid, and neutralized using alkali before immunostaining. Some (oo)cysts might still remain attached to the beads after acid treatment (McCuin & Clancy 2005). IMS is the only purification technique prescribed by USEPA. Water quality may also affect the IMS efficiency; high turbidity may reduce the recovery efficiency(Campbell & Smith 1997; Hsu & Huang 2007; Stanfield et al. 2000). Dissolved iron in water may also reduce the recovery efficiency down to 0% (Yakub & Stadterman-Knauer 2000).

The complex of beads and (oo)cysts is dissociated by the addition of acid. This is another crucial step, where (oo)cysts may remain attached to the beads after acid treatment (McCuin & Clancy 2005). Some modifications have been attempted to eliminate this loss and improve the recovery rate. By adjusting the pH to neutrality, the oocysts recovery rate was increased by 26% (Kuhn et al. 2002), and some studies have suggested to increase both the bead and sample ratio as well as incubation time (Carey et al. 2006; Hsu & Huang 2007; Hsu & Huang 2001; Pezzana et al. 2000). This should be balanced by the cost of the reagents, and recent studies have revealed that the same recovery rate can be acquired by using a less amount of beads (Utaaker et al. 2015) as long as the matrix is within standards of turbidity. If

the sample is of high turbidity this can be overcome by increasing the sample ratio by dividing the eluate (ISO 2006).

However, these methods do not give parasite identification to genotype level and as environmental samples often contain debris and inhibitors hampering PCR, it makes source tracking and further identification of the possible source cumbersome.

4.1.2 Tools for detection of *Cryptosporidium* oocysts and *Giardia* cysts in vegetables

Methods for analysing fresh produce for *Cryptosporidium* and *Giardia* have been under development since the 1990s, when outbreaks associated with these parasites were identified, and have had a great importance in western countries. Their impact on public health in water-borne outbreaks have rushed scientists to the drawing table, and the availability of reagents developed for testing water made the task more comprehensible. Different methods have been described in the literature, with varying recovery efficiencies and reproducibility, though all includes the four main procedures:

I) Elution.
II) Concentration of eluate.
III) Isolation/separation of (oo)cysts from sample debris.
IV) Detection.

Bier et al (1991) were the first to publish a method for finding *Cryptosporidium* oocysts on fresh produce. This method was based on sonification in detergents and concentration by centrifugation. However, this method had a recovery efficiency of only 1% from seeded cabbage and lettuce leaves.

Robertson and Gjerde (2000) modified a protocol for water samples, and the method based on elution, sonification, concentration, IMS separation and IFAT detection, gave a recovery efficiency of 40% for *Cryptosporidium* oocysts and around 70% for *Giardia* cysts on a variety of fresh produce(lettuces, strawberries, cabbage and carrots). Bean sprouts were also tested, but the recovery efficiency for this particular produce was significantly lower. This study elevated the recovery efficiency as well as identified sample details of importance for recovery efficiency.

Cook et al (2007) found recovery efficiencies of both *Cryptosporidium* and *Giardia* to be around 36%. This study used internal controls, which further validated the results, and these were further established by Rzezutka et al (2010), who used the same method as Cook, though with a different supplier of beads and only looked for *Cryptosporidium* oocysts. This study found a recovery efficiency between 4% and 47% for a variety of vegetables (onion, leek, lettuce, cauliflower and cabbage). This publication gave a semi – independent verification of efficiency, though with lower recovery rates than the developing lab.

Amoros et al (2010) used the method published by Cook et al (2007) and found recovery efficiencies for leafy vegetables. This was significant as an independent verification of an already published method, though with lower recovery efficiencies; 30% lower for *Cryptosporidium* oocysts and approximately 50% lower for *Giardia* cysts.

Utaaker et. al. (2015) verified the method already outlined by the Veg-I-Trade project, which was based on the previous publications. A round-robin test further validated the protocol, proving recovery efficiency to be satisfactory when reproduced by several participating laboratories, with a 53% mean recovery of *Cryptosporidium* oocysts and 33% mean recovery of *Giardia* cysts. This method also reduced the quantity of IMS beads, the most expensive component per analysis, and simplified the steps of analysis from previous studies.

4.2 Diagnostic tools for detection of *Cryptosporidium* oocysts and *Giardia* cysts in faeces

Many methods are available for diagnosing and detecting giardiasis and cryptosporidiosis, as mentioned in the general presentation. These include direct visualisation of cysts, trophozoites and oocysts in faeces, and indirect methods that identify *Giardia* and *Cryptosporidium* antigens in faecal samples. at specialized laboratories samples may be tested with immunofluorescence assay tests (IFAT) and/or molecular methods such as PCR.

4.3.1 Immunofluorescent antibody testing (IFAT)

Immunofluorescence antibody testing uses antibodies that bind specifically to an antigen, in this case the same or similar surface wall glycoproteins on *Giardia* and *Cryptosporidium* (oo)cysts as used for IMS. These antibodies have a pre-bound fluorescence molecule, allowing them to be observed under the right wavelength of fluorescence. For this project, a commercial test kit was used (Aqua-glo, Waterborne Inc., New Orleans).

4.3.2 DAPI staining (4´6-diamino-2-phenylindole)

DAPI visualizes nuclear DNA in both living and fixed cells, and can be used to determine the number of nuclei and assess gross cell morphology, in combination with IFAT, these stains are useful tools for determination of presence of *Giardia* cysts and *Cryptosporidium* oocysts in a sample and their morphology.

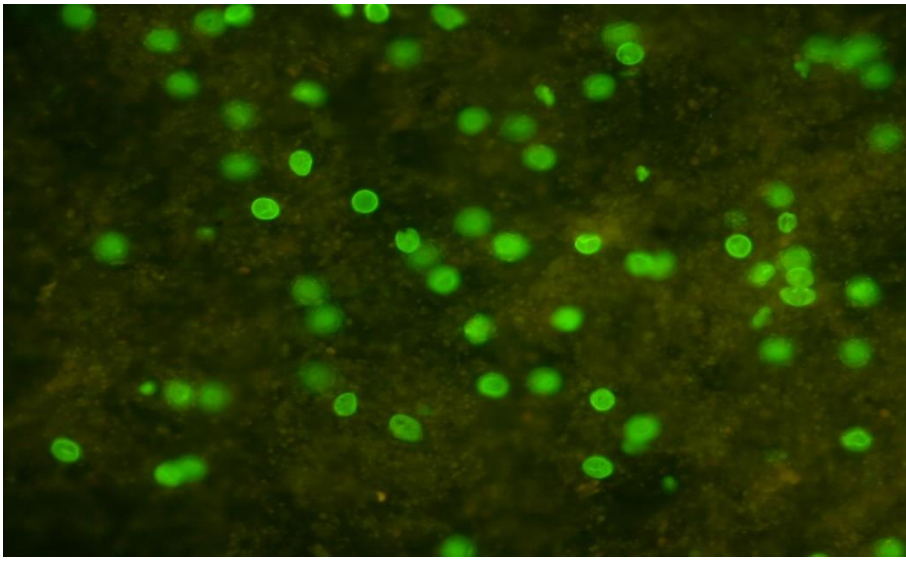


Figure 11. *Cryptosporidium* oocysts visualised using IFAT

4.3 Viability assessment methods

Determination of viability of *Cryptosporidium* oocysts and *Giardia* cysts is a field of high importance as it takes only a few infective (oo)cysts to establish infection. Dead (oo)cysts have no public health significance, as they are not able to complete their life cycle. The current standard methods for detection does not include viability testing or genotyping, which is crucial knowledge in terms of establishing the potential for transmission or outbreaks. Robertson and Gjerde (2007) concluded that the gold standard for determining infectivity are bioassays with neonatal mice. However, this is an invasive method which requires resources and sacrifice of animals, and there are additional methods including simple dye inclusion and exclusion which should be further tested and approved for standard use in a laboratory setting, making a simple, cheap and practical approach for assessing infectivity. In view of public health, a (oo)cyst that is incapable of initiating infection has as little public health significance as a dead and disintegrated (oo)cyst. A viable (oo)cyst, defined as “capable of reproducing under appropriate conditions” (Robertson et al. 1998) can be infective or non-infective, and a non-viable (oo)cyst is always non-infective.

Different techniques have been developed for assessing the viability and/or infective potential of *Cryptosporidium* oocysts and *Giardia* cysts:

Infectivity studies:

The neonatal mouse model has been used for assessing infectivity in disinfection studies, and gnotobiotic pigs and human volunteers have been used for assessment of infective dose and drug efficacy. This method is expensive, time-consuming and labour intensive, and strict ethical standards must be met. Also, not all host species are suitable (e.g. *C. hominis* is not infective to mice) and the extrapolation of results from an animal model to a human situation must be evaluated carefully. In addition, infectivity studies are not suited for assessing small numbers of (oo)cysts, and are therefore unsuitable for environmental samples. Nonetheless, such studies are the only method providing definitive evidence of infectivity of (oo)cysts that have been exposed to different environmental or chemical pressures, and thus remains the golden standard (Robertson & Gjerde 2007).

Cell Culture

Cell culture models have been proposed as an alternative for the detection of infectious *Cryptosporidium* oocysts. The method implies basically the *in vitro* excystation with sporozoites enabled to invade cultured cell monolayers. Invasion can be assessed and quantified by a variety of methods, such as PCR techniques, immunofluorescence and *in situ* hybridisation. This is a technically demanding technique, and high variability has been demonstrated, including unexpected stimulatory responses. (Rochelle et al. 2002; Rochelle et al. 2005). Nonetheless, results under certain conditions have shown to be equivalent to those from infectivity studies while being cheaper, faster and without ethical drawbacks, and it can be applied to assess the infectivity of low numbers of oocysts (Di Giovanni & LeChevallier 2005). However, the cell culture method alone cannot be used to enumerate the number of oocysts present in environmental samples as it is not clear if the presence of one infectious oocyst will infect one or more cells in the cell culture. Although cell culture can detect infectious *C. parvum* and *C. hominis*, it is not certain which other *Cryptosporidium* species can infect cell monolayers. Also, cell cultures cannot be used to assess the infectivity of *Giardia* (Burnet 2012).

Detection of mRNA

mRNA detected from heat shock protein synthesis in viable (oo)cysts (Stinear et al. 1996). The heat shock proteins are known to be synthesized in stressed organisms. Thus, when (oo)cysts are exposed to a thermal shock, the induction of a heat shock response provides an index of viability (Abbaszadegan et al. 1997). Decay of mRNA transcripts for β-tubulin (Widmer et al. 1999), amyloglucosidase (Jenkins et al. 2000) and other markers have been used similarly. In addition, as real-time PCR allows the continuous monitoring of amplicon formation throughout the reaction, quantitative aspects could also be studied. This method has also been compared to vital dye staining and found comparable in measuring viability (Bertrand et al. 2009), but comparisons with gold-standard infectivity have only given marginal correlation for some assays (Jenkins et al. 2002).

Fluorescent *in situ* hybridization

This method is based on the theory of rRNA breakdown following cell death. By targeting a specific sequence of rRNA in *Cryptosporidium* oocysts and *Giardia* cysts, FISH probes are

designed only to label potentially infective or recently inactivated (oo)cysts (Dorsch & Veal 2001; Vesey et al. 1998). In situ hybridization seems to correlate well with in vitro excystation, which though overestimates infective potential, and it seems not to correlate well with cell cultures or infectivity studies(Jenkins et al. 2002). In addition, some studies have indicated prolonged rRNA stability after oocyst death (Fontaine & Guillot 2003), and research during the Sydney water enquiry demonstrated high FISH signals from oocysts killed by radiation (McClellan 1998).

In vitro excystation

Various protocols have been described in which exposure to given conditions will activate excystation in potentially infective (oo)cysts. It has also been used in combination with real-time PCR to quantitatively estimate (oo)cyst viability (Bertrand et al. 2009). However, in vitro excystation requires relatively high numbers of (oo)cysts (Robertson & Gjerde 2007; Sauch 1991) and it is considered to be a subjective method due to the presence of partially emerged trophozoites and sporozoites (Labatiuk et al. 1991). It may be useful for supporting results obtained from other assays.

Vital dye inclusion/exclusion

Vital dye techniques developed by (Campbell et al. 1992; Schupp & Erlandsen 1987) are the ones most frequently used, and includes inclusion or exclusion of (oo)cysts stained with DAPI (4`, 6-diamidino-2-phenylindole) and PI (Propidium iodide), and differential interference contrast microscopy (DIC) for assessing viability of individual (oo)cysts. DAPI stains the nucleus sky blue, while PI can only pass through damaged cell membranes and intercalates with the nucleic acids of injured and dead cells to form a bright red fluorescent complex. An intact cyst wall may be insufficient to induce an infection in the host (Bertrand et al. 2009), and this technique has been found to frequently overestimate infective potential of (oo)cysts. On the other hand, this is a cheap and readily implemented method, and can be applied a small number of, or even single (oo)cysts, and can divide the parasites into different categories based on staining and content of the (oo)cyst when combined with sophisticated optics. Due to the simplicity and availability of vital dye techniques, this method was used to assess viability of *Cryptosporidium* oocysts and *Giardia* cysts on lettuce in this thesis.

4.3.3. Molecular tools for detection of *Cryptosporidium* and *Giardia*

Molecular tools for genotyping and subtyping gives a deeper insight into host specificity of *Cryptosporidium* species and subspecies as well as *Giardia* Assemblages and subassemblages and their transmission. Identification of species infecting humans and animals and the proportion of infection attributable to each species in various geographic, epidemiologic and socioeconomic settings is a valuable tool in unravelling foci of infection, such as infection or contamination sources and characterization of transmission dynamics in communities.

In the polymerase chain reaction (PCR), primers complementary to *Giardia* and *Cryptosporidium* gene sequences are used together with DNA polymerases that will amplify small amounts of *Giardia* or *Cryptosporidium* DNA present in the sample, to enable detection. Both generic and genotype specific primers and protocols have been published. Conventional PCR often ends with running the samples on an agarose gel by electrophoresis, and visualizing bands of DNA with different types of staining, e.g. cyanine dyes. The size of the product is determined with a ladder that is loaded simultaneously with the samples. Different methods have been developed for real time identification and quantification of DNA, and by adding fluorescently labelled nucleotide probes that are complementary to the amplified sequence it is also possible to distinguish between different genotypes (Elwin et al. 2014). Genotyping is more commonly performed in relation to research, as it may provide more certain answers regarding epidemiology, focus of infection and source tracking.

4.4 Material and methods used in this study

The Materials and Methods described in this section are divided into two sections. The first part is concerned with the Materials and Methods used in the lab-based experimental part of the study, in which all the work was conducted in the Parasitology Laboratory at NMBU. This includes the work described in Papers I and II.

The second part is concerned with the Materials and Methods concerned with the surveys conducted in India, and describes the sampling sites and sample collection methods, the lab work conducted in the Parasitology Laboratory at PGIMER, and the lab work associated with the survey work conducted in the Parasitology Laboratory at NMBU (referring sometimes to the Materials and Methods described in Section 1 – Experimental Studies).

Statistical approaches used are described at the end of this section and apply to both experimental work (Section 1) and survey work (Section 2).

4.4.1 Section 1 – Experimental Work

Source of *Cryptosporidium* oocysts and *Giardia* cysts used in experimental studies

1) Positive control *Cryptosporidium* oocysts and *Giardia* cysts in buffered mixed aldehyde solution for use with Waterborne Kits produced by Waterborne Inc., New Orleans, LA, USA was used for the initial spiking of vegetable and water samples when these protocols were incorporated into the thesis work.

2) Pre-counted *Cryptosporidium* oocysts and *Giardia* cysts from EasySeed™ produced by Biomérieux Industry was used for the interlaboratory trials when establishing the reduced-cost approach, as well as establishing the recovery rates for the water sample analysis.

3) For the viability studies, *Cryptosporidium parvum* oocysts isolated from faeces of naturally infected calves by salt flotation by Hønsvall and Robertson (2017) and live *Giardia* cysts belonging to Assemblage B from the H3 isolate (gerbil source) suspended in PBS ordered from Waterborne Inc.

Enumeration of *Cryptosporidium* and *Giardia*

For the live *Giardia* cysts and *Cryptosporidium* oocysts used in the viability studies, the

(oo)cysts were diluted into individual stock solutions. 10 µL aliquots from the stock was pipetted on a multispot microscope slide (C.A. Hendley (Essex) LTD), stained with a monoclonal antibody (mAb; Aqua-glo, Waterborne Inc, New Orleans, USA) before enumeration and further dilution.

Spiking studies

In the experimental studies where recovery of *Cryptosporidium* oocysts and *Giardia* cysts were evaluated, the (oo)cysts from EasySeed™ were already counted by the manufacturer. When inter-laboratory trials were initiated, in order to save costs, the spikes sent were equivalent to 50 % EasySeed™ spikes that had been prepared in-house. This was done by shaking individual EasySeed™ spike vials vigorously and then dividing into two aliquots, each containing 50 % of the original spike.
Thus, each participant was sent a seeding sample containing approximately a 50 % EasySeed™ size spike and an empty EasySeed tube that had been cleaned by soaking in 15 % sodium hypochlorite overnight, then washed in hot soapy water three times, and then rinsed, and contained only water (same volume as for the spike) – that is a negative control. None of the participants were aware that only one sample contained parasites.

When the method for water samples was assessed, only in-house laboratory trials was done, and EasySeed™ (oo)cysts were used. This was assessed by performing the method according to the ISO-standard for water analysis, but replacing the reagents as described for the reduced-cost approach.

For the viability assessments, the matrix to be evaluated, in this case lettuce leaves, were sprinkled with approximately 50 000 *Giardia* cysts and 50 000 *Cryptosporidium* oocysts. The (oo)cysts, diluted in distilled water from stock solution to a volume of 100 µl, were spread on the lettuce leaves in aliquots of 20 µl using a pipette.

Assessment of viability

Viability was assessed based on morphology and inclusion and exclusion of the vital dyes 4',6-diamino-2-phenylindole (DAPI) and propidium iodide (PI), as described by Campbell et al., (1992). In order to identify the parasites eluted from the lettuce, a monoclonal antibody (mAb; Aqua-glo, Waterborne Inc. New Orleans, USA) was added to the suspension in the final 15 minutes of the staining procedure. The stained sediment was examined in suspension (with the cover slip on the microscope sealed with nail varnish to avoid drying) by fluorescence microscopy using a Leica DMCB microscope equipped with a UV filter block (350-nm excitation, 450-nm emission) for DAPI and a green filter block (500-nm excitation, 630-nm emission) for PI. Nomarski (differential interference contrast) optics on the same microscope was used to examine morphology of individual cysts and oocysts.

The cysts and oocysts were evaluated and categorised according to exclusion or inclusion of the different stains and their morphology. Empty or shrunken (ghost) cysts and oocysts were identified under Nomarski optics, containing no nuclei or shrunken residues thereof. They were also non-refractile, apart from the residual body when present.

PI+ cysts and oocysts fluoresce bright red under the green filter block; this fluorescence varies from distinct points corresponding to the sporozoite / trophozoite nuclei, to a more diffuse fluorescence within the cyst or oocyst. Cysts and oocysts were categorised as DAPI+, PI- if they did not include PI (as described above), but the nuclei of the sporozoites/ trophozoites fluoresced a distinctive sky blue under the UV filter block. Cysts and oocysts that were neither PI+, nor "ghosts", and which showed either rim fluorescence or absence thereof under UV filter block were considered DAPI-, PI-. The interpretation of these various appearances is described in Table 3. For the purposes of this study, DAPI+, PI- parasites and DAPI-, PI- parasites were summed together as viable or potentially viable.

Table 3. Categorisation of cysts and oocysts according to inclusion and exclusion of vital dyes, DAPI and PI, and morphological parameters

Cyst / oocyst categorisation	Vital dye inclusion		Nomarski microscopy	Viability designation
	DAPI	PI		
Ghost	No	No	Shrunken, deformed, empty shell, lacking contents	Dead
PI+	Yes	Yes	May be deformed, contents	Dead
DAPI+, PI-	Yes	No	Good morphology, contents	Viable at assay
DAPI-, PI-	No	No	Good morphology, contents	Viable, but may need further trigger to excyst

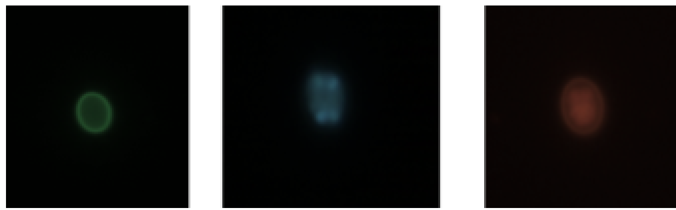


Figure 12. IFAT, DAPI and PI – positive *Cryptosporidium* oocysts (Picture acknowledgement Lucy J. Robertson and Ane Holbø).

4.4.2 Section 2 – Survey Work

Study area

Chandigarh is a city located northwest in India. It is the joint capital of its neighbouring states, Punjab and Haryana, and is a relatively new dwelling, as its construction started in the early 1950s. It is situated at the foot of the Shivalik hills, and is known as the “city beautiful”, as it is one of the greenest cities of India, with many parks and recreational areas, and the services provided by Chandigarh Administration and the Municipal corporation are considered as a role model for other urban cities. Chandigarh is also one of the fastest growing cities in India, with a current population of over a million inhabitants (JNNURM 2006).

The city was designed by a French architect, LeCorbusier, in the 1950s, and reflects the modernism movement that arose in Europe around that time. The city’s outline is considered as head, lungs, heart and limbs, but it seems that these organs of bricks and humans are currently undergoing an uncontrollable hypertrophy. The original plan was to divide the city into phases, and these phases are further divided into sectors. Sectors 1 to 30 were designed for a population of 150 000 inhabitants, and this area is referred to as phase I. Sectors 31 to 47, phase II, and were supposed to house 350 000, meaning the population density in this area would be 4 times higher than phase I. Now, with the population increase, phase III is on the rise, and this is also an area with a planned higher density than those of the previous two. The rise in population has given the “city beautiful” quite a challenge in terms of the rehabilitation of slum colonies, presence of urban villages with unregulated growth in the planned sectors, the presence of unauthorised settlements, limited land for future growth and development, as well as how to address the problems of waste management, water supply, and general infrastructure in a city with an accelerated population growth. These are a few of the many trials for a city with a population of the very rich to the very poor, and which seems to be growing into a fractal city pattern of economic and social differences (Chandigarh Administration 2016).

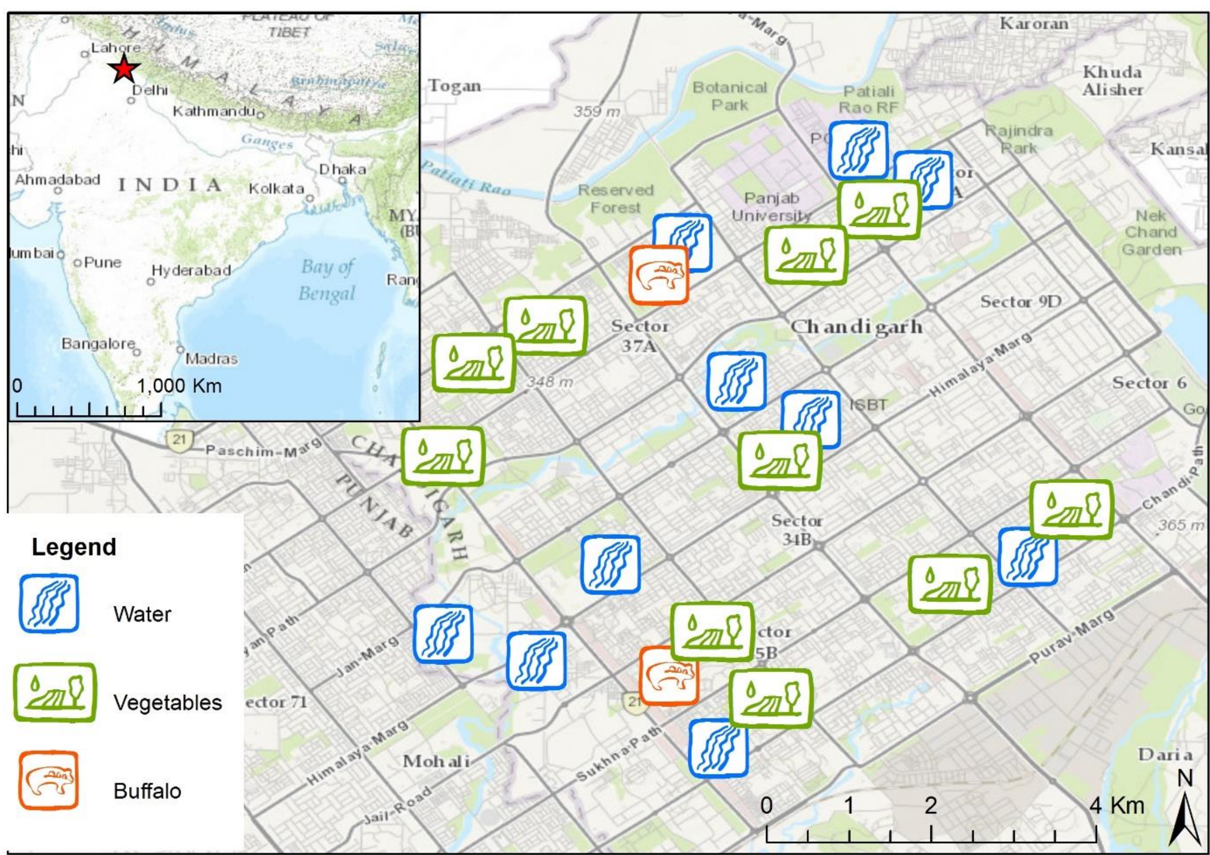


Figure 13. Schematic overview of Chandigarh (Picture acknowledgement, Gabriel Gati).

Collection of samples

The collection of samples required prolonged stays in India and fieldwork with researchers from the collaborating lab. A detailed sampling plan for each of the sample types (animal faeces, water samples, fresh produce samples) could not be developed prior to my visits to India as there was insufficient information, and I was reliant on my collaborators at PGIMER both in terms of providing the correct information and for the pragmatic requirements of obtaining the necessary permission and assisting with the practical duties. It was important for the project to obtain enough samples to have usable results and to be able to compare results across factors of interest such as seasonality and socio-economic strata. For the latter, this involved collecting samples from the different Phases of Chandigarh, as described in Section 4.4.2.1, as these generally reflect the socio-economic status of the different resident groups. It had been anticipated that I would also collect all sample types according to the results of the human data collected by PGIMER in WP1 of the Para-Clim-Chandigarh

project; however, due to the delay in PGIMER conducting their work, I had to collect my samples without taking their data collection and results into consideration.

As PGIMER does not routinely analyse drinking water or fresh produce for parasitic protozoa, samples were partly processed and analysed at PGIMER in India, then shipped to Norway for completion of the analyses at the Parasitology Lab at NMBU. This meant that samples had to be preserved to ensure that they could be successfully and safely shipped between India and Norway.

Animal faeces, collection and storage of samples prior to shipment

As contact with animal faeces in India is traditionally a job for the so-called "untouchables", the Dalits, which are the lowest caste in India and who have traditionally been stigmatized and isolated, collection of faecal samples proved to be initially difficult to approach. Although the caste system has officially been abolished, in practicality it still exists in some areas, and the social stigma of touching animal faeces continues.

Faecal samples from goats, cattle, buffaloes, and dogs were collected from farms, households, parks and wasteland. The samples were collected non-invasively either from the animal or directly from the ground as fresh, morphologically consistent stools in some study areas. For samples taken from animals, only one sample per animal was obtained due to practicality and logistics. For samples collected from the ground, it was not always possible to determine whether the samples were from different animals or repeated faecal samples from the same animal or group of animals.

Samples were put directly into containers and transported to the laboratory at PGIMER within maximum two hours. Here they were immersed in 2.5 % potassium dichromate, a cheap preservative which allows future molecular diagnostics, for preservation and before shipment and processing. Various storage media and approaches have been associated with lack of PCR amplification. Potassium dichromate was chosen as preservative for faecal samples in this study, as they seem to keep both *Giardia* cysts and *Cryptosporidium* oocysts intact and preserves DNA well during storage (Wilke & Robertson 2009).

Water samples, collection, initial processing, and storage of samples prior to shipment

For the first batch of water samples collected, the initial processing was based on the flocculation method as described by (Vesey et al. 1993) was applied. However, due to poor recovery rate and logistics, as the remaining sample to be transported to Norway was typically 300ml, the method was abandoned and the results from these samples were not included in the final article submitted for publication.

Figure 14. Collection of water samples.

Water samples were mostly collected from these public sources, with samples collected into 10 L plastic jerry cans from different public water collection points (either public stand posts or water tanks) throughout the city. A total of 71 water samples were collected, with 39 samples collected from phase I sectors, 12 samples were collected from phase II, and 20 samples were collected from phase III and non-sectorial villages surrounding the city. After collection, the containers were taken directly to the Parasitology laboratory at PGIMER and processed immediately (within 2 h) for subsequent shipping.

For subsequent water samples, I brought a portable pump (Watson-Marlow 520 Bp Profibus) and filters to the Parasitology laboratory at PGIMER, and the samples were filtered through Millipore Isopore membrane filters with a pore size of 2 µm. The filters were stored refrigerated until transportation to Norway, where the downstream procedures were performed (see Section 4.5.2). This made the logistics and practicalities easier to handle, as

the filter from one sample containing 10 L of potable water could be kept in water in a 50ml centrifuge tube before the next steps were conducted.

The method for detecting *Cryptosporidium* oocysts and *Giardia* cysts in water samples consist of 3 steps; I) concentration, II) separation III) detection. The concentration step was performed in India.

Concentration

Concentration of (oo)cysts is a crucial step which may influence the recovery rate. The concentration technique used in this thesis work was membrane filtration, where in general, (oo)cysts are concentrated on a filter, and the concentration step was performed in the collaborating lab. 10L samples were collected and filtered and the filter was retained in a 50ml centrifuge tube with distilled water before refrigeration and transportation to Norway.

Figure 15. Filtration of water samples at PGIMER.

Vegetable samples, collection, initial processing, and storage of samples prior to shipment

Vegetables are sold at different markets in Chandigarh, which are arranged at different weekdays for individual sectors. Street vendors usually have a push-cart or a cart connected to a bike, which enables them to roam different areas of the city in search of customers, and supermarkets are unevenly dispersed throughout the city. Samples for this study was bought directly from these retailers, put in a plastic bag and transported to the laboratory for further processing.

Vegetable samples

The principle of analysing vegetable samples is quite similar to that of water samples, and can be divided into four stages; I) Elution, II) concentration, III) Isolation and IV) detection. Only step I) will be discussed here in detail, as step II), III) and IV) are equivalent to the water protocol.

Elution

Elution is the removal of parasites from the surface of the vegetable into a liquid medium that can be more readily manipulated. Vegetables were put into filtered stomacher bags and eluted with 200 ml 1M Glycine buffer, a buffer readily available and easy to make on-site. The stomacher bag was then manipulated for 4 minutes by hand, as no stomacher was available in the lab, and the eluate transferred into 5 centrifuge tubes of 50 ml each, as the stomacher bag was rinsed with 50ml distilled water to improve parasite recovery. The tubes were centrifuged at 1550 rfg for 10 minutes, and the supernatant was removed using a sterile 10ml plastic syringe. The remaining pellets were vortexed and combined per sample and stored at 4°C before transportation to Norway.

4.5 Completion of sample processing at the Parasitology Lab at NMBU

Samples arriving at NMBU were kept refrigerated until processing. All samples were processed as soon as possible after arrival.

4.5.1 Detection of *Cryptosporidium* oocysts and *Giardia* cysts in faecal samples

The samples preserved in potassium dichromate were rinsed with phosphate buffer before further processing. Canine, bovine and goat faecal samples were processed by McMasters for analysis for helminths. 3 grams of faeces was homogenized with distilled water (57 ml per 3 grams) with a blender. After sieving through 125 µm porosity steel mesh, allowing the parasite eggs and (oo)cysts to move through while removing larger particulates, the suspension was poured into plastic tubes and centrifuged at 3000 rpm for 3 minutes after which the supernatant was decanted. The tube was added flotation solutions, either NaCl or NaCl/ZnCl salts, which due to their density gradient allows parasite eggs and (oo)cysts to float to the top while debris sinks to the bottom, creating a plane to focus on. The solution was pipetted into a McMaster counting chamber, which allows for quantitative evaluation of the number of eggs / oocysts per gramme of faeces.

Using a plastic loop, 20µl of sample material from the concentrated pellet was placed in a microscope slide, dried and fixed with methanol. After the methanol dried off, 10-20 µl of FITC conjugated monoclonal antibodies (Waterborne Inc., USA) were applied and the slides incubated in a humidifier at 37°C for 30 minutes. Slides were investigated under a fluorescent microscope (Leica MLD) with filters for viewing FITC.

To increase the yield of *Cryptosporidium* oocysts and *Giardia* cysts, pelleted goat feces was run by an IMS protocol, which significantly improved the recovery of parasites compared to the traditional smear (Utaaker 2017, unpublished results). In addition, retrieving (oo)cysts from IMS has proven to give more positive results when DNA is extracted and PCR performed (Coklin et al. 2011).

4.5.2 Methods for isolation of *Cryptosporidium* and *Giardia* from water and vegetable samples.

The methods used for analysing water and vegetable samples are derived from the ISO-standards, with modifications to reduce the costs. The modifications were made in terms of reducing some of the reagents and replacing other with cheaper options. Both methods have the basic concepts of filtration, either through micropores in the case of water, or filtered stomacher bags filled with elution buffer for vegetables, then concentration for creation of a pellet with concentrates of debris from the samples, and immunomagnetic separation using magnetic beads to separate parasites from debris, following the final step of examination using fluorescent dye and DAPI staining.

Concentration of water samples in Norway

In Norway, the filter was washed with membrane buffer as described in method 15553:2006, and the eluate was centrifuged at 1550 rfg for 10 minutes to create a pellet from water debris.

Preparation of concentrated vegetable samples before separation

The centrifuge tubes containing the concentrated eluate was added SDS before vortexing and centrifugation at 1550 rfg for 10 minutes. The supernatant was removed using an aspirator and the pellet was transferred to an L10 tube together with buffers and magnetic beads. The procedure is further described in section 4.1.1.

Separation of parasites from water and vegetable samples

The separation procedure was performed using immunomagnetic separation (IMS), which involves the attachment of (oo)cysts with magnetic beads coated with parasite-specific monoclonal antibodies. The continuous mixing of beads in the water sample causes separation of (oo)cysts present in the samples from debris.

The pellet was resuspended into a L10 tube and filled with buffers and magnetic beads, and the tube was rotated for approximately one hour in order to create a cyst/oocyst complex with the beads. The beads were washed into an Eppendorf tube, and the complexes were dissociated using 0.1M HCl and vigorous vortexing. After dissociation, the remaining solution was pipetted onto a hydrophobic welled slide where NaOH had been added to neutralize the pH, and the slide was left to dry overnight.

Microscopy and detection of *Cryptosporidium* oocysts and *Giardia* cysts from water and vegetable samples

Dried samples were fixed with methanol and stained with FITC-conjugated monoclonal antibodies (mAbs) against *Cryptosporidium* oocyst walls and *Giardia* cyst walls (Aqua-glo^{TM}, Waterborne^{TM} Inc, USA) and nuclei were stained with the fluorogenic DNA intercalator 4´, 6 diamidino-2-phenylindole (DAPI) according to Smith et al., (2002). Samples were mounted with M101 No-Fade Mounting Medium, then each slide was covered by a glass coverslip and viewed promptly by fluorescent microscopy.

Microscopy was performed on a Leica DCMB microscope (x 20, x 40 and x100 objectives), equipped with Nomarski differential interference contrast (DIC) optics. A blue filter block (480 nm – excitation, 520 nm – emission) was used for the detection of cysts and oocysts labelled with FITC-conjugated mAbs, and a UV filter block (350-nm excitation, 450-nm emission) was used for investigating DAPI-staining.

Each well was scanned systematically in an up-and-down or side-to-side manner at 20X, and *Cryptosporidium* oocysts and *Giardia* cysts were enumerated. When brilliant apple-green fluorescing ovoid or spherical objects within the appropriate size range for *Cryptosporidium* and *Giardia* were observed, magnification was increased to 40X, and the UV filter block was used for visualization of DAPI staining. Each (oo)cyst was recorded as DAPI-negative or DAPI-positive according to the presence of internal light blue staining.

Nomarski (DIC) objectives were used to examine morphological characteristics of the (oo)cysts.

A sample was considered positive if the (oo)cyst(s) exhibited typical fluorescence, with correct shape and size, and being DAPI-positive. If internal contents were lacking, but the morphometry was correct and the structure had a typical fluorescence, the (oo)cysts were described as “putative”, as they lacked sufficient characteristics for definitive identification.

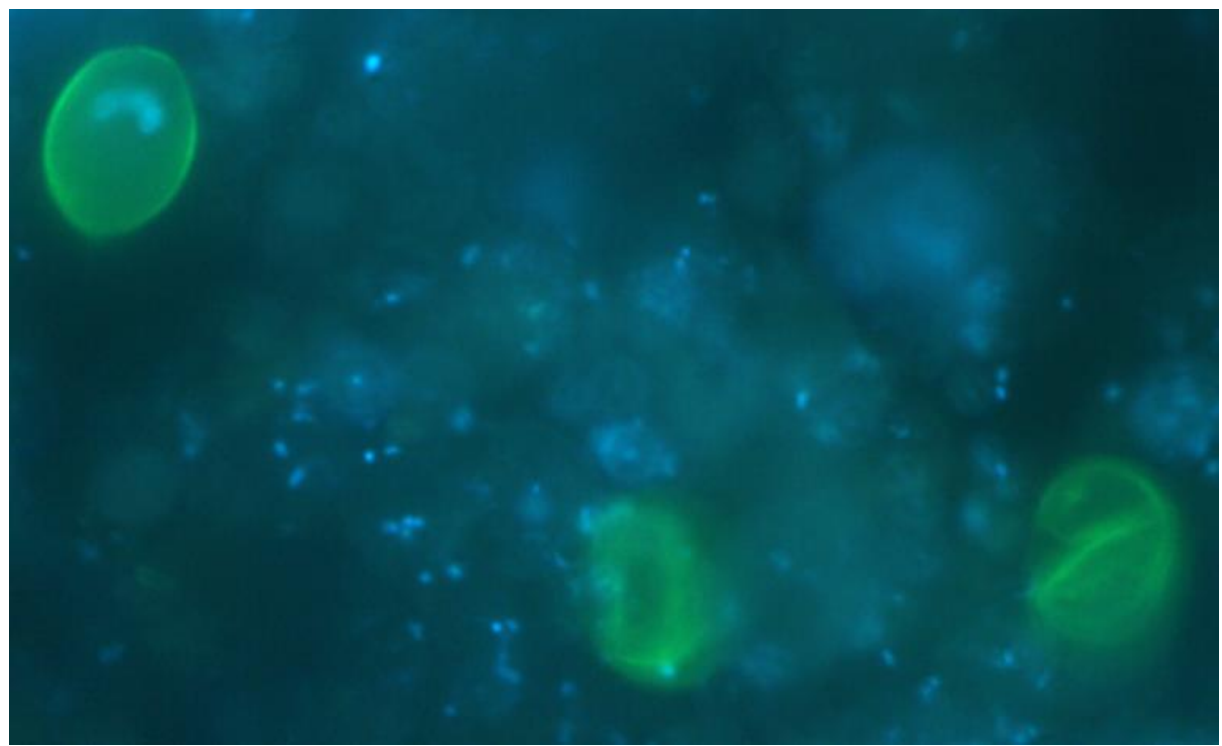
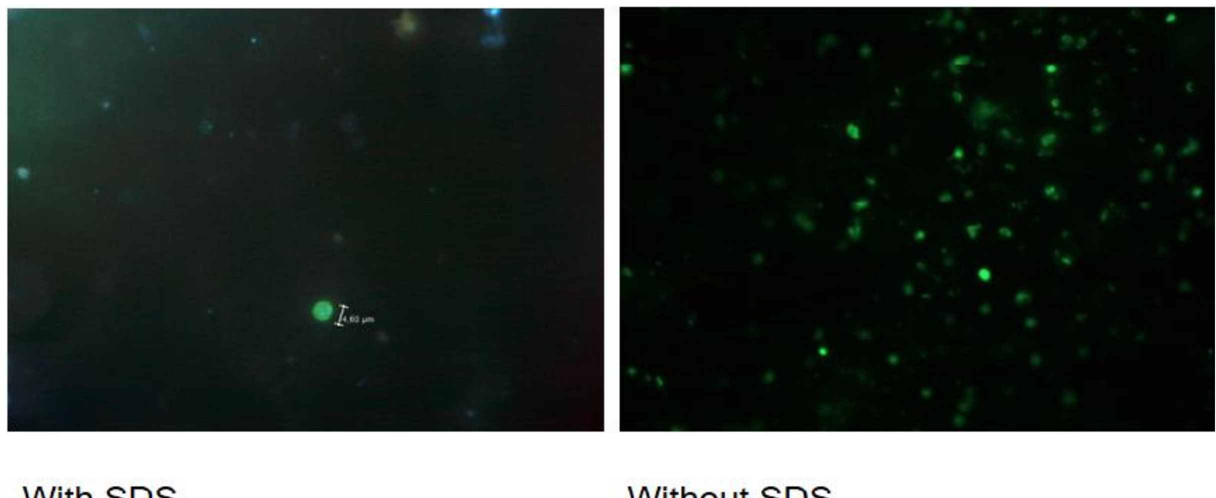


Figure 16. *Giardia* cysts where one has visible DAPI – staining. The cyst on the right has ruptured and no nuclei is visible.

The ISO standards can be found in (ISO 2006) for water and (ISO 2016) for fresh leafy green vegetables and berry fruits. The modified methods for vegetables and water are further described in paper I and IV in this thesis.

The glitch of the gloop – stepping into the vegetable matrix

Due to lag in transport and poor storage conditions, some of the vegetable samples developed bacterial growth, and some had even turned mouldy. By applying a detergent in the sample, sodium dodecyl sulfate (SDS), the slimy consistency of the sample was to some extent inhibited, enabling the magnetic beads to adhere more easily to the parasites in the matrix. This was tested by spiking some discarded samples and using them as a test matrix; the application of detergent improved the recovery rate significantly, as well as improving the view in the microscope as well as occluding debris was minimised (see figure 16). The application of detergent is also included in the reduced-cost protocol (Utaaker et al. 2015).



With SDS　　Without SDS

Figure 16. Debris in vegetable sample stored over a longer period with and without SDS added.

4.6. Molecular methods

4.6.1 DNA isolation of faecal samples

Disruption of the oocysts and cysts is performed to make DNA more available for extraction. Techniques that can be used may include freeze-thawing, boiling, bead-beating and lysis (Checkley et al, 2015).

For *Cryptosporidium* oocysts and *Giardia* cysts, DNA was isolated using a QIAmp mini kit (Qiagen GmbH). This was performed either directly on the faecal pellet, on purified (oo)cysts after IMS. The protocols followed the manufacturers` instructions with slight modifications; cysts / oocysts were first mixed with 150 µl of TE buffer (100mM Tris and 100 mM EDTA) and incubated at 90°C for *Giardia* cysts and 100°C for *Cryptosporidium* oocysts for 1 hour before an overnight proteinase K lysis step at 56°C and spin column purification. DNA was finally eluted in 30 µl of PCR-grade water and stored at 4°C.

4.6.2 DNA isolation from water and vegetable samples

Following microscopy, *Cryptosporidium* oocysts and *Giardia* cysts were retrieved from positive slides and DNA was prepared according to Robertson et al. (2009). Briefly, the coverslip from each slide was carefully removed and retained, whilst 25 µl aliquots of AL lysis buffer (Qiagen GmbH, Germany) were added to the slide wells, which were then scraped using a sterile scalpel blade. The buffer and scrapings were pipetted into a microcentrifuge tube. This process was repeated four times, and then the coverslip was replaced onto the slide that was then re-screened. For each slide no cyst or oocysts could be detected after the procedure.

The contents of each microcentrifuge tube containing slide scrapings were re-suspended in Tris-EDTA buffer and held at 100°C for *Cryptosporidium* oocysts and 90°C for *Giardia* cysts for one hour, before the DNA was isolated using QIamp DNA mini kit (Qiagen GmbH), using an overnight step at 56°C.

4.6.3 Conventional polymerase chain reaction (PCR)

PCR may allow detection of very small quantities of parasite DNA within a sample. As it generates many copies of the target DNA, the result can then be sequenced, and this genetic code used to characterise an isolate and determine its taxonomy and phylogenetic relationship to other isolates (Lymbery and Thompson, 2012).

The PCR reaction occurs in a welled chamber where two primer sets, which bind specifically the target DNA; RNAse – free water, bovine serum albumin (BSA), which stabilises the enzymes and reactions, DNA polymerase, which is the enzyme creating the copies of DNA, and a mixture of DNA nucleotides. PCR products are visualised after separation across a 2% agarose gel using electrophoresis, and can be compared with a DNA ladder to determine fragment lengths.

For *Cryptosporidium*, use of the first generation genotyping tools has decreased as the techniques have become more specific to target species, though one of these targets, the *Cryptosporidium* oocyst wall protein (COWP) is still used as a confirmative genotyping method due to the robustness of the technique. The small subunit ribosomal ribonucleic acid (SSU rRNA) gene is a widely used target in PCR, and can be used to distinguish between species, which can then be subtyped at the 60 kDA glycoprotein (gp60) gene in the case of *C. parvum* and *C. hominis* (Checkley et al. 2015; Shirley et al. 2012).

For *Giardia*, four commonly targeted genes for molecular characterisation of an isolate were utilised in these studies; Small subunit ribosomal ribonucleic acid (SSU rRNA), beta-giardin (bg), glutamate dehydrogenase (gdh) and triosephosphate isomerase (tpi). These genes vary in the substitution rate per nucleotide, thus their ability to distinguish isolates at different levels of genetic detail vary from SSU rRNA being useful to distinguish species, to the more heterogenous tpi gene where isolates at sub-assemblage levels can be typed (Feng & Xiao 2011).

Positive control for molecular methods were obtained by isolating DNA from *Cryptosporidium parvum* oocyst isolated by Hønsvall and Robertson (2017) and Holbø (2017), and *Giardia* cysts belonging to Assemblage B from the H3 isolate (gerbil source) suspended in PBS ordered from Waterborne Inc.

PCR protocols used in this study are given in Table 4.

Table 4. PCR conditions for detection of *Giardia* and *Cryptosporidium.*

Locus	bp	Primer	Cycle conditions		References
Giardia					
Small Subunit (SSU) rRNA	292	1st amplification	96 °C, 5 min		Hopkins et al., (1997)
		F: 5'-CATCCGGTCGATCCTGC-3'	96 °C, 30 sec		Read et al.,(2002)
		R: 5'-AGTCGAACCCTGATTCTCCGCCAGG-3'	59 °C, 40 sec	40 x	
			72 °C, 40 sec		
			72 °C, 7 min		
	175	2nd amplification	96 °C, 5 min		
		F: 5'-GACGCTCTCCCCAAGGAC-3'	96 °C, 30 sec		
		R: 5'-CTGCGTCACGCTGCTCG-3'	55 °C, 40 sec	50 x	
			72 °C, 30 sec		
			72 °C, 7 min		
Triosephosphate Isomerase (TPI)	605	1st amplification	95 °C, 10 min		Sulaiman et al., (2003)
		F: 5'-AAATYATGCCTGCTCGTCG-3'	94 °C, 45 sec		
		R: 5'-CAAACCTTYTCCGCAAACC-3'	50 °C, 45 sec	45 x	
			72 °C, 60 sec		
			72 °C, 10 min		
	563	2nd amplification	95 °C, 10 min		
		F: 5'-CCCTTCATCGGNGGTAACTT-3'	94 °C, 45 sec		
		R: 5'-GTGGCCACCACVCCCGTGCC-3'	50 °C, 45 sec	45 x	
			72 °C, 60 sec		
			72 °C, 10 min		
Glutamate Dehydrogenase (GDH)	755	1st amplification:	94 °C, 2 min		
		F: 5'- TTCCGTRTYCAGTACAACTC-3'	94 °C, 30 sec		Caccio et al., (2008)
		R: 5'- ACCTCGTTCTGRGTGGCGCA-3'	50 °C, 30 sec	35x	
			72 °C, 1 min		
			72 °C, 7 min		
	530	2nd amplification:	94 °C, 15 min		
		F: 5'- ATGACYGAGCTYCAGAGGCACG T-3'	94 °C, 45 sec		
		R: 5'- GTGGCGCARGGCATGATGCA-3'	54 °C, 45 sec	50 x	
			72 °C, 45 sec		
			72 °C, 10 min		

Gene	Size (bp)	Primers	PCR conditions	Cycles	Reference
Glutamate Dehydrogenase (GDH)		1st amplification:	94 °C, 15 min		Read et al., (2004) Robertson et al., (2006)
		F: 5’-TCAACGTYAAYCGYGGYTTCCGT-3’	94 °C, 45 sec	50 x	
		R: 5’-GTTRTCCTTGCACATCTCC-3’	54 °C, 45 sec		
			72 °C, 45 sec		
			72 °C, 10 min		
Beta Giardin (BG)	753	1st amplification:	95 °C, 15 min		Caccio et al., (2002) Lalle et al., (2005)
		F: 5’-AAGCCCGACGACCTCACCCGCAGTGC-3’	94 °C, 30 sec	35 x	
		R: 5’-GAGGCCGCCCTGGATCTTCGAGACGAC-3´	60 °C, 30 sec		
			72 °C, 60 sec		
			72 °C, 10 min		
	511	2nd amplification	95 °C, 15 min		
		F: 5’-GAACGAGATCGAGGTCCG-3’	95 °C, 30 sec	40 x	
		R: 5’-CTCGACGAGCTTCGTGTT-3’	53 °C, 30 sec		
			72 °C, 60 sec		
			72 °C, 10 min		
Cryptosporidium					
SSU rRNA	860	1st amplification	95 °C, 15 min		Xiao et. al., (1999)
		5’-GGAAGGGTTGTATTTATTAGATAAAG-3’	94 °C, 45 sec	50 x	
		5’-AAGGAGTAAGGAACAACCTCC A-3’	55 °C, 45 sec		
			72 °C, 60 sec		
			72 °C, 10 min		
COWP	769	1st amplification	94 °C, 5 min		Yu et. al., (2009)
		5´-ACCGCTTCTCAACAACCATCTTGTCCTC-3	94 °C, 50 sec	30 x	
			55 °C, 55 sec		
		5´-CGC ACC TGT TCC CAC TCA ATG TAA ACC C-3`	72 °C, 50 sec		
			72 °C, 10 min		
Gp60	850	1st amplification	95 °C, 3 min		Sulaiman et. al., (2005) Glaberman et. al., (2002)
		5´-ACCGCTTCTCAACAACCATCTTGTCCTC-3`	94 °C, 45 sec	35 x	
			50 °C, 45 sec		
		5´-CGC ACC TGT TCC CAC TCA ATG TAA ACC C-3`	72 °C, 1 min		
			72 °C, 10 min		
	400	2nd amplification	95 °C, 3 min		
		5´-TCCGCTGTATTCTCAGCC-3`	94 °C, 45 sec	35 x	
		5´-CGAACCACATTACAAATGAAGT-3`	50 °C, 45 sec		
			72 °C, 1 min		
			72 °C, 10 min		

4.6.4 Sanger sequencing, sequence alignment and analysis

Purified PCR products were sent to external laboratories where conventional Sanger DNA sequencing was performed. This is a process involving a similar system to the PCR described above, though some nucleotides have fluorescent labels and result in cessation of the amplification process. The solution is then passed through a reading chamber, with the order dependent on the size of the amplified sequence, and the fluorescent labels are counted by a machine. In this way, a chromatogram of the resulting fluorescent labels is created, representing the sequential nucleotides of the original PCR products, which can be read in the form of a DNA sequence.

The sequences obtained were manually checked for consistency using the program Genious®, and the resulting sequence compared against other reported sequences in the GenBank database. For gp60 positive samples, the 5` end of the sequence was manually checked for tandem repeats of the serine-coding trinucleotides TCA, TCG and TCT, and checked for any repetitive sequences for determination of subtype family in addition to comparing other reported sequences in the GenBank database. When the genotype or subtype was established, the sequence was sent to NCBI with and given an accession number (See tables 5,6,7,8 and 9).

4.7 Statistics

4.7.1 Experimental studies (paper I, II and IV)

Reduced-cost approach

Vegetables

For inter-laboratory trials, the accuracy of the method is described by sensitivity and specificity parameters, that is the percentage of known positive test material that were correctly defined as such (sensitivity) and the percentage of known negative test correctly identified as such (specificity).

For calculating the percentage of false negatives and mean recovery efficiencies, the confounding factors listed by Scotter et al. (2001) were considered for exclusion of results (temperature abuse during shipment; clear deviations from the method in the testing laboratory; questionable laboratory performance). Determinations were performed according to the following equations (Cook et al. 2006):

$$\%\ \text{false negatives} = \frac{\text{Number of samples where threshold level not met}}{\text{Number of samples to which parasites were added}} \times 100$$

$$\%\ \text{false positives} =$$

$$\frac{\text{Number of samples to which parasites were not added, but parasites were detected}}{\text{Number of samples to which parasites were not added}} \times 100$$

Concordance, which here is defined as the chance that two identical test materials sent to different laboratories will both be given the same results, and accordance, the qualitative equivalent of repeatability, meaning the chance that two identical test materials analysed by the same laboratory under standard repeatability conditions will both give the same result for the trials were defined and calculated according to Langton et al (2002).

Water

(Statistical analysis in this study was performed by Eystein Skjerve)

For comparing the results for the modified water analysis protocol, recovery efficiencies of the spiked *Cryptosporidium* and *Giardia* by the modified method were compared with those obtained by the ISO 15556 method by performing linear regression.

Survival study

(Statistical analysis in this study was performed by Eystein Skjerve)

Relative viability at each time point were obtained by normalizing the data to the initial viability by using the equation: *Percentage viability =* $(N_t/N_0) \times 100$

Where N_t is the number of viable parasites at time t (of 100 parasites), and N_0 is the number of viable parasites at time 0 (of 100 parasites).

Survival was analysed using a linear regression model, with method as a categorical variable and time (log10 hours) as a continuous predictor was utilised, and a follow up logistic regression comparing the viability data of the parasites on the lettuce from initial contamination point until final sampling point. Standard graphical methods were used to assess mode fits and residual patterns using the statistical software Stata /SE/14 for Windows, StataCorp, College Station, TX.

Comparing methods of *Giardia* cyst isolation

Two methods were analysed using 40 randomly selected samples and compared using Fisher's exact test, based on categorical data in a two-by-two contingency table.

4.7.2 Survey studies

A database of results was created in excel and parametric and non-parametric (ANOVA and Mann-Whitney U-tests) were used to compare mean and median values. Contingency table analysis (chi-square and Freeman-halton) was used to test for associations between positive results, prevalences in different areas, difference in positive finding between seasons and socioeconomic layers and other factors. Statistical significance was considered for p values < 0.05.

For comparison of *Giardia* prevalence according to location (urban/peri-urban), Students T-test was used.

5. Results and general discussion

5.1 Experimental studies

Reduced cost modified version for analysing fresh produce and water for *Cryptosporidium* and *Giardia*

The initial studies, that focused on modifying the technique for analysis of fresh produce for occurrence of *Cryptosporidium* oocysts and *Giardia* cysts, resulted in a method of 80% reduced cost per analysis, but with comparable mean recovery efficiencies (53% for *Cryptosporidium* oocysts and 33% for *Giardia* cysts) to the standard method. These modifications certainly cut the costs of this project, and may be of use to other research projects or laboratories on constrained budgets as the supplementary reagents can easily be obtained. The inter-laboratory trial provides independent confirmation of the method's applicability, and is an important step in such processes of identifying methods that work in different laboratories, as new approaches that produce improved recovery efficiencies may not always transfer well to other labs. One downside of the independent laboratory testing of the method was only possible for labs in Europe. Although we did try to include labs further away (Canada, Malaysia), we found that unstable storage during shipping of the samples made the effort useless. This does emphasise that correct storage of reagents is important, and this may also be problematic in some situations, for example when duration of shipping of reagents is prolonged.

Based on the results obtained, this modified approach could be a useful tool for surveillance projects in both developed and developing countries, where efforts should be directed towards monitoring the production chain, and where detection and genotyping could be an important step for finding glitches and evaluating preventive measures in the production chain.

In addition, by using the same approach and reagents, we were able to analyse water samples using this protocol; again, this also reduced the cost of this study while apparently not affecting recovery efficiency, and this approach could also hopefully benefit other research projects.

On the more costly end, by utilizing immunomagnetic separation beads for faecal samples from goats, a significantly higher recovery of *Giardia* cysts was achieved. This separation technique may be used in projects where *Giardia* prevalence is assessed, but where it is only possible to obtain one sample per animal. As *Giardia* cyst shedding is intermittent, a single negative sample does not immediately state that the individual is not shedding cysts, and this separation technique may give a more true reflection of the actual prevalence. However, as a diagnostic tool from clinical samples it is of low cost-benefit, as it is only likely to identify samples that would otherwise be missed where cyst excretion is very low (and thus less likely to be of clinical relevance). In addition, tests that identify antigens (rather than cysts), such as immunochromatographic procedures, may be another approach to identifying animals infected by *Giardia.* On the positive side, use of IMS may enable an efficient, although costly, approach for isolating cysts or oocysts for downstream molecular analyses with fewer inhibitors being present.

Survival of *Giardia* cysts and *Cryptosporidium* oocysts on fresh produce in the household

The results of the study described in Article II confirmed that although *Giardia* cysts seem to survive when kept in cool, moist conditions, exposure to ambient temperatures results in relatively rapid die off. In contrast, the relatively more robust *Cryptosporidium* oocysts did not seem to be affected by storage temperature. Based on these results, it would seem likely that unless fresh produce is consumed rapidly after contamination events, or is kept refrigerated until consumption, *Giardia* cysts will not remain viable and thus be unable to cause infection when ingested. As fresh produce in India is mostly sold by traditional retailers, who sell their fruits and vegetables outdoors at ambient temperature, and as only 30% of Indian households own a refrigerator (Mahambare 2017), the likelihood of foodborne giardiasis may be generally low, when contamination has occurred early in the production chain, and is of greatest relevance when contamination occurs directly prior to consumption, possibly in the kitchen, which are again linked to the importance of basic hygiene knowledge. However, due to the robustness of the oocysts and the lack of control in the production chain, the likelihood and impact of foodborne cryptosporidiosis may be considerable.

5.2 Survey studies

5.2.1 Environmental surveys

Occurrence of *Cryptosporidium* and *Giardia* on vegetables from different retailers in Chandigarh

It has been stated that foodborne transmission of these protozoan parasites is an under-recognized but important emerging issue, especially in developed countries, due to the increasing globalization of food trade, international travel, increased number of immunocompromised and other susceptible individuals as well as changes in consumer habits, and that fresh produce imported from developing countries are of great concern (Dixon 2016). In my opinion, the elimination of this threat should start at the source, as the threat is just as valid, if not more so, for the populations in developing countries as the developed. The possible sources of contamination have been listed as poor personal hygiene of workers during production, harvesting, packaging or transport, or by indirect contamination of produce at the farm level through faecally contaminated water in irrigation, mixing of pesticides, or washing of produce, hands or equipment, as well direct contamination by the salesperson, food handler and consumer. Control measures, such as properly treated water, health monitoring of workers, improved on-farm sanitation, and restricted access of livestock and other animals to crops and surface water have been proposed. However, these measures are difficult to implement overnight in a country where animals traditionally roam freely. Basic hygienic measures seems to be the key in preventing contamination of fresh produce, a food item not traditionally eaten raw, though with the impact of western habits, culinary choices are now more diverse in urban areas of India, and a contaminated salad just as infective in these regions as the one flown into developed countries.

From the survey conducted in my study, a total occurrence of contamination of 11% was found (Table 5), with a low median (oo)cyst count in positive samples. Noteably, outliers in *Cryptosporidium* oocyst counts ranging from 350 and over 1000 per sample was found on fresh produce bought at a supermarket and from a vendor in a slum area. From these results, it seems that the traditional retailers keep their stock relatively free from

contamination. One of the reasons for this can be that the salespersons aim to retain and expand their customer base and not therefore do not offer them vegetables that appear unfresh and unpalatable, or that the consumers may later associate with being unwell. Also, the introduction of supermarkets in a developing country, where traditional markets currently have a strong foothold, is challenging. In addition, trying to implement a model that is based on rigid hygienic routines where large masses of produce are transported through a production chain, into a country where the infrastructure is unstable seems like the wrong end to begin.

The genotyping results from my study (Table 5, Article III) revealed both zoonotic and canid-specific genotypes, and the source of contamination can only be guesses as the production chain is not properly monitored. The value of surveying fresh produce itself in these settings may also be questionable, as the main contamination problems seem to occur in different links of the production chain itself that are not properly monitored. Breaking the production chain into its separate links, and monitoring over the whole length of the chain may produce useful information regarding where contamination is most likely to occur, and thus indications of where interventions should be introduced and their potential effects be monitored.

Table 5. Presentation of the main results from the vegetable study.

Fresh produce	**season**	**location**	**Genotyping results**	**Sample ID**	
	Winter/Spring	Phase I / vendor	Assemblage D	KY967232	
	Monsoon	Phase II / vendor	Assemblage A	KY967233	
	Winter/Spring	Phase II/ Supermarket	*C. parvum*	KY 967230, KY 967231	
	Winter/Spring	Phase II / Supermarket	*C. parvum*	KY967229	
Results			**occurrence *Cryptosporidium* oocysts**	**Occurrence Giardia cysts**	**Total Occurrence**
		Median counts on contaminated produce	4 oocysts	2 cysts	
			5 % (n=13/284)	6 % (n=17/284)	11% (n=284)
	No seasonal variance	Phases/location: no significant difference Retailer: significant difference			

Occurrence of *Cryptosporidium* and *Giardia* in drinking water in Chandigarh

In Chandigarh, there was no routine monitoring of contamination of drinking water with protozoans. This procedure requires reagents specialized for this purpose as well as trained lab personnel, and the parasitology lab in PGIMER was specializing in diagnosing and treating human infections, not contaminated water or environmental samples. In the initial stage of the project, a flocculation method was used on the first fourteen samples, but this had poor recoveries and made logistics more problematic due to the weight of the end product after sedimentation, so this approach was abandoned. However, one *Giardia* cyst was found in one sample using this method, although this finding is not part of the submitted final article as it was considered more of a preliminary result.

From the 71 water samples collected and analysed, a total occurrence of contamination of 22.5% was found (Table 6). This is a high number, in the sense that one fifth of the water in Chandigarh may contain *Cryptosporidium* oocyst and/or *Giardia* cysts. Although only two of the samples were successfully genotyped, one of those contained a canid-specific genotype and was thus not infectious to humans. Nonetheless, this finding shows that contamination may occur from any source, and routines for clean water may not be properly executed in Chandigarh, despite this city having a highly developed infrastructure compared with other Indian cities. Interestingly, the samples from the area with the second lowest population density was found to be significantly more likely to be contaminated. The sample with the highest number of parasites was collected in a slum colony, and contained >1000 *Giardia* cysts. Water scarcity has become a problem in many Indian cities, and Chandigarh is no exception. To solve water shortages, water tankers are driven into slum areas and the main city when water supply is short. One of the main goals described by the Municipal Corporation is to have a continuous water supply for the population of Chandigarh, but achieving this aim seems to be far away, as the work to increase potable water supply has been held up for nearly a decade (Hindustan Times 2017), and the city currently is suffering from water shortages due to hot summers ad leakages, and is now implementing penalties on residents misusing potable water (Sehgal 2017).

Table 6. Presentation of the main results from the water study.

Water samples	Season	Location	Genotyping result - *Giardia*	GenBank ID	
	Winter/Spring	Phase II	Assemblage B	MF 150151	
	Winter/Spring	non-sectorial village	Assemblage C	MF 150152	
Results			**Occurrence *Giardia***	**Occurrence *Cryptosporidium***	**Total prevalence**
	no seasonal variance in contamination	Significantly Phase II p=0,001	14.1% (n= 10/71)	9.9% (n=7/71)	22.5% (n=16/71)

5.2.2 Domestic and stray animal surveys from Chandigarh

Prevalence of *Cryptosporidium* and *Giardia* in extensively reared goats

Goats are kept by those who already need to supplement an already scarce income in developing countries, and India is no exception. The samples in this study were collected from slum areas and peri-urban settlements where the infrastructure is poor, and sanitary basics are often lacking. This was reflected in the relatively high prevalence of *Giardia* and the genotyping results of the goats, where the majority of genotypes were zoonotic. From the 207 samples collected, 34.3% were *Giardia* positive, and from those successfully genotyped, 68% belonged to zoonotic genotypes. Only one sample was *Cryptosporidium* positive (0.5%) (Table 7, Article V). As samples were only collected during the winter season, no seasonal variance could be assessed, and the low prevalence of *Cryptosporidium* oocysts in faeces could be due to that most of the animals from which we obtained samples from were adults. As noted in the data, the general prevalence of goats shedding *Giardia* cysts was high, and the high proportion of zoonotic genotypes may reflect the conditions both humans and goats were living in. Simple routines may be implemented in these settings that could contribute towards raising the human-animal barrier. In this backyard goat setting, sharing households apparently means that also parasites are shared, and the implementation of simple routines, such as more separate housing and education in hygiene when handling animals, may help in reducing infections in goats and their owners.

Table 7. Presentation of the main results from the backyard goat study.

Goat samples	location	Genotyping results *Giardia*	Sample ID	Genotyping result *Cryptosporidium*	Sample ID
	Urban	Assemblage B	MF069062		
	Urban	Assemblage E	MF069058		
	Urban	Assemblage A	MF069057		
	Urban	Assemblage B	MF069047		
	Urban	Assemblage A	MF069052		
	Urban	Assemblage A	MF069051		
	Urban	Assemblage E Assemblage C	MF084938, MF069071		
	Urban	Assemblage E	MF084935, MF069070		
	Urban	Assemblage A	MF069056		
	Urban	Assemblage E	MF069059		
	Urban	Assemblage D	MF069055		
	Peri-urban	Assemblage A	MF069054		
	Peri-urban	Assemblage E	MF084936, MF095054, MF106203, MF069072		
	Peri-urban	Assemblage E	MF084934, MF095052		
	Peri-urban	Assemblage B	MF 095053, MF069053		
	Peri-urban	Assemblage B	MF069066		
	Peri-urban	Assemblage B	MF069064		
	Peri-urban	Assemblage B	MF069060		
	Peri-urban	Assemblage E	MF084937		
	Peri-urban	Assemblage B	MF069050		
	Peri-urban	Assemblage A	MF069068		
	Peri-urban	Assemblage A	MF069067		
	Peri-urban	Assemblage E	MF069065		
	Peri-urban	Assemblage B	MF069063		
	Peri-urban	Assemblage B	MF069061		
	Peri-urban	Assemblage A	MF069069		
	Peri-urban	Assemblage A	MF069049		
	Peri-urban	Assemblage A	MF069048		
	Peri-urban			*C. ubiquitum*	MF124820
Results					
	Location not significant				
	Summation of results	***Giardia***		***Cryptosporidium***	**Total prevalence**
	Prevalence:	34.3%		0.5%	34.7% (n= 207)
	Cyst shedding:	Median cyst count: 275			
	Distribution of genotypes:	Ass. A: 36%, Ass B: 32%	Ass. E, C and D: 32%		

Occurrence of *Cryptosporidium* and *Giardia* in bovines

The samples collected in this study came mostly from farms, or gaushalas, so-called cow sanctuaries where cattle living beyond their productive years are being kept. Welfare organizations donate money to these farms, and they are cared for by employees. The cattle are kept in enclosed housing, and they do not live in close contact with humans to the same extent as backyard goats. From the 294 samples collected, a prevalence of 8.2% for Giardia and 2.4% for Cryptosporidium was found (table 8, Article VI). The difference in close living with humans for cattle sampled here compared with goats may explain the difference in *Giardia* Assemblages found in the two separate studies, where both the prevalence of *Giardia* cysts and occurrence of zoonotic assemblages was lower in cattle. Interestingly, and somewhat in contradiction with the results regarding *Giardia* Assemblages, *Cryptosporidium* subtypes correlating with previous findings in humans in the same area was also found in calves, indicating that even though the prevalence is low, parasites are shared between bovines and humans in Chandigarh.

Table 8. Presentation of the main results from the bovine study.

Calf faeces	Season	Location	Genotyping result *Cryptosporidium*	Sample ID	Genotyping result *Giardia*	Sample ID	
	Winter	Peri-urban			Assemblage B, Assemblage E	MF399205, MF459679	
	Winter	Peri-urban	*C. bovis* *C. parvum*, subtype IIdA15G1	MF399200, MF535626			
	Winter	Peri-urban	*C. parvum,* subtype IIdA15G1	MF399201, MF459681			
	Winter	Peri-urban			Assemblage A	MF163432	
	Winter	Urban			Assemblage E	MF399204, MF459678	
	Monsoon	Urban			Assemblage E	MF163433	
	Monsoon	Urban			Assemblage E	MF399203	
	Monsoon	Urban			Assemblage E	MF399206	
	Monsoon	Peri-urban	*C. bovis*, *C.parvum* subtype IIdA15G1	MF399202, MF535627			
	Monsoon	Peri-urban	IIdA15G1 and	MF535999,	Assemblage B	MF459680	
Results			**Prevalence *Cryptosporidium***		**Prevalence *Giardia***		**Total prevalence**
	not significant	not significant	2.4% (n=7/294)		8.2% (n= 24/294)		9.5% (n=28/294)

Occurrence of *Cryptosporidium* and *Giardia* in dog samples

From the 212 samples collected, a 24% prevalence of Giardia was found, with a significantly higher prevalence during the winter season and in the more densely populated part of Chandigarh (Table 9, Article VII). Although dog ownership in India rising, and India has been projected to be the fastest growing global pet market, in general, stray and roaming dogs are not highly regarded in India. This distance between humans and dogs could also be seen when comparing the genotyping results, where only 10.4% of the *Giardia* cysts had zoonotic potential. However, such a distance between humans and dogs in terms of *Giardia* has also been demonstrated in many countries of the world where dog ownership and affection for dogs is much higher, and a review article on *Giardia* in pet animals concluded that the zoonotic potential of *Giardia* in pet animals is probably minor (Ballweber et al. 2010). Nevertheless, in these countries it can be speculated that the human-dog barrier is due to elevated awareness and environmental cleanliness. Thus, the data regarding *Giardia* in dogs in Chandigarh contrasts with results from the goat study in terms of Assemblage distribution, and with the results from the cattle study in terms of occurrence. The difference of positive samples according to socioeconomic layers of the city is interesting, and canid-specific assemblages have also been found in the environmental samples, indicating that dogs are able to roam and leave their mark without human interference, further emphasizing the need for sustainable control of the dog population in Chandigarh.

Table 9. Presentation of the main results from the dog study.

Dog faeces	season	location	Genotyping results - *Giardia*	Sample ID	
	Monsoon	Phase II	Assemblage A	MF153909	
	Monsoon	Phase I	Assemblage D	MF281089	
	Monsoon	Phase I	Assemblage C	MF153397	
	Winter	Phase I	Assemblage C	MF153910	
	Monsoon	Phase II	Assemblage C	MF281090	
	Monsoon	Phase I	Assemblage C	MF153911	
	Winter	Phase II	Assemblage C	MF281091	
	Monsoon	Phase I	Assemblage C	MF281098	
	Winter	Phase II	Assemblage C	MF281092	
	Monsoon	Phase II	Assemblage D	MF281093	
	Winter	Phase II	Assemblage C	MF281094	
	Monsoon	Phase I	Assemblage B	MF281095	
	Monsoon	Phase I	Assemblage C	MF153912	
	Winter	Phase II	Assemblage C	MF281096	
	Monsoon	Phase II	Assemblage E	MF281097	
	Winter	Phase I	Assemblage C	MF153913	
	Winter	Phase II	Assemblage C	MF153914	
	Monsoon	Phase II	Assemblage D	MF153915	
	Winter	Phase I	Assemblage D	MF153916	
Results	**Seasonal variance**	**Location /positive samples**	**Distribution of *Giardia* genotypes**		**Total prevalence**
	Significant	Significant	Assemblage C and D: 84.4%		
			Assemblage A and B: 10.4 %		
			Assemblage E: 5.2%		24% (n=212)

Combined conclusions from all the survey-based studies

As these various survey studies have revealed, implementation of some actions towards general runoff, hygiene, sanitation and simple education could reduce the burden of parasite contamination, and thus of infection in both humans and animals. It seems that animals may have a role in the spread of both *Cryptosporidium* and *Giardia* and in maintaining the lifecycle in Chandigarh, although to different extents with different animals, and analysis of the genotypes revealed that humans may be the group who can be considered to bear responsibility for the cycle. In addition, and not considered here, wild animals in close contact with humans may also play a role, as the worshipped and public fed maquace monkeys in Chandigarh showed a high prevalence of zoonotic *Giardia* genotypes (Debenham et al. 2017).

However, from the holy cow to the family goat, zoonotic potential was found in domestic animals in close contact with humans, with goats being of particular importance. However, no significant zoonotic potential was found in the dog study, and as stray dogs are usually avoided by humans, this was not a surprising result.

5.3 Limitations and challenges experienced in the study

The application of molecular methods in samples traveling across the globe

Polymerase chain reaction (PCR) is one of the most extensively tested and widely used techniques for investigating the origins of waterborne protozoan parasites. PCR is not only often used to identify pathogens in a complex environment, but by targeting specific gene sequences, PCR assays can be used to distinguish between different sub-groups (assemblages or subtypes) of the same species or identify different species within a genus.

Although PCR is considered to be highly sensitive and accurate, and for pathogenic bacteria is in the process of replacing traditional methods, for parasites it has limitations. Although the potential for false positives should not be ignored, and may result from laboratory contamination, false negatives are a more common problem. These may occur due to low recovery of DNA during the extraction process, especially in the case of environmental samples with a low number of (oo)cysts and a high degree of organic debris in the sample. In addition, inhibitors like polysaccharides, polyphenols, pectin, xylan and chlorophyll may be extracted along with the (oo)cysts and thus hamper the PCR reaction in the case of environmental samples (Wei et al, 2008). Faecal samples are also particularly difficult, as they are complex and highly variable matrices that contain an enormous quantity of DNA from bacteria and cells other than the target DNA (Wilke and Robertson, 2009). In addition, in the absence of a normal cellular processes, endogenous endonuclease activity, bacterial degradation and spontaneous depurination results in relatively rapid breakage of DNA strands, and DNA degradation may continue due to oxidative action and accumulation of molecular cross-linkages (Deagle et al, 2006).

Many of the samples collected in this study, both faecal samples and water/vegetable samples, contained low numbers of (oo)cysts, and as environmental samples are very diverse, they may also have contained a number of PCR inhibitors, such as debris, fulmic and humic acid, metal ions and polyphenols which may be extracted along with the parasites during DNA isolation, and hamper DNA amplification during the PCR reaction (Abbaszadegan et al. 1993; Ijzerman et al. 1997). Although faecal samples from a symptomatic individual often have a higher concentration of (oo)cysts and thus DNA, faeces also contain inhibitors such as complex polysaccharides, bile salts, lipids and urate (Schrader et al. 2012). In my

study, the faecal samples were not necessarily obtained from animals with clinical symptoms, and my study was to investigate potential reservoirs of human infection, thus they also include non-symptomatic low excretors. In general, the samples collected in this study were from a plethora of sources and settings, and many inhibitors may have been extracted along with the DNA.

Due to practical and logistical issues between the collaborating laboratories, the period between collection and examination of faecal, drinking water and vegetable samples was in some cases prolonged. Storage of *Giardia* cysts has proven to cause alterations in membrane morphology, intense vacuolization as well as damage of its wall (Santos et al. 2015), and a decline in sporozoite ratio during storage has also been found in *Cryptosporidium* oocysts (Dawson et al. 2004). Parasites may have degenerated during storage and the formation of other microorganisms during the storage and transport period may have had a deteriorating effect on the DNA in terms of both degeneration and formation of inhibitors. Especially the environmental samples proved hard to genotype.

Opportunities and Challenges

There are numerous challenges when working in developing communities where resources are not readily available, and the cultural differences are sometimes hard to comprehend for a foreigner, so the sparsely understood and underdocumented relations of socioeconomic, cultural, religious, educational and bureaucratic nuances cannot be investigated with confidence in this work. However, these variables were influential in shaping the view of society. As a female veterinarian collaborating with native farmers and collecting faecal samples, challenges in obtaining samples sometimes went beyond communication and caused misunderstandings, though almost always with the outcome of collaboration and mutual understanding in the end. Traditionally the domestic animals are viewed as a part of the family and culture, they are greatly cared for, and the myriad of cultural differences were hard to rectify during the relatively short duration of field work.

Even though there were difficulties in logistics and transport of over a thousand samples with DNA (and inhibitors) it was possible to apply refined molecular tools to unravel zoonotic relationships. Minimal quantities of sample are required for molecular screening and

characterization of parasites, which makes air transportation between collaborating laboratories possible, and the chemical properties of potassium dichromate in solution make them a safe transport vehicle for biological material. Moreover, the DNA for all parasite species in whole faeces preserved in these solutions can remain stable even at temperatures up to 40°C for prolonged periods before analysis, which proved to be a useful trait.

The hurdle of applying molecular-based tools does not lie in having a specialized laboratory on-site, or time delays in sample processing, but in the considerable cost of equipment, reagents and personnel associated with processing the samples. By dividing the labour to two workplaces; collection and preparation for transport at PGIMER with further processing at NMBU, where the laboratory already had the necessary equipment and resources, both collection and analysis were possible to perform.

The ability to selectively and sensitively detect and genetically characterize parasitic stages directly from faeces and the environment has served as a major advantage for studying the epidemiology of parasites within populations and geographical areas. This is especially valuable information for a country where these relationships have not been explored to a great extent yet, and where these diseases have a generally higher prevalence. Molecular tools in combination with classical parasitological and epidemiological methods to detect, diagnose and genetically characterize parasites are imperative in studies aiming to assess sources and foci of infection.

6. Concluding remarks and future perspectives

Although we know that *Cryptosporidium* and *Giardia* can be foodborne and waterborne, and both have zoonotic potential, the bulk of the studies that address this issue and attempt to determine the importance of these transmission routes and sources of infection have been conducted in developed countries. Although developed countries have had many more reported outbreaks of foodborne and waterborne cryptosporidiosis and giardiasis, and the zoonotic potential has been quite extensively investigated, such studies from less developed countries are lacking. The epidemiology of these infections is likely to differ considerably between such different settings, and, as the infrastructure is considerably less robust in developing countries, I would assume that the potential for transmission via environmental contamination would be greater. Thus, there is a notable gap in our understanding of the transmission routes and infection sources for these parasites in countries such as India, and the work described in this thesis goes someway to addressing these questions in a city in northern India.

The experimental, lab-based studies enable the development and validation of analytical procedures based on standard methods, but at considerably reduced costs, thereby enabling me to generate reliable data in a challenging environment. The survival studies went some way to providing data indicating one reason why foodborne giardiasis may be less of a concern than foodborne cryptosporidiosis, particularly in a setting where fresh produce is not often refrigerated.

From the results in the survey studies, there seems to be a correlation between how close humans interact with the animals around them, and the occurrence of zoonotic protozoans in animal faeces. This can only be amended by human intervention on transmission routes, and to be able to do that, awareness is the first step towards prevention.

The survey study from this project indicated that clean water seems to be a privilege for the population in Chandigarh living in the socioeconomic higher layers of the city. No seasonal variance was found in the water samples, and this may be due to a backdrop of constant contamination and poor surveillance, or the fact that the seasons during the sampling period was somewhat not as predicted.

Based on the results generated during the work described in this thesis, it is clear that if fresh produce in India is to be both produced and consumed as happens in western countries, then the production chain needs to be evaluated and improved. In the Apni Mandi system, the farmer may be more accessible, but the hazards in a system where the farmer is responsible for all steps in the trade should be closely supervised, and hazards in the production chain should be pointed out to those engaged in this type of trade. There is a clear need for implementation of such systems as Good Agricultural Practice (GAP), Good Manufacturing Practice, (GMP) and Good Handling Practice (GHP) (FDA 1998). In the supermarket model, the origin of the wares may be less simple to track, and thus relevant factors in how the produce have been grown, harvested, or processed. This makes results of analysis of fresh produce less valuable if the sources and critical control points are to be evaluated and improved. In addition, my study showed that drinking water in Chandigarh has relatively high occurrences of protozoan parasites, and more effective surveillance studies with emphasis on source tracking through genotyping is a useful tool in epidemiological investigations. Although the results of my study may act as an indicator, the results cannot be extrapolated to all cities in India. However, the potential for contamination in such settings is clear, and thus the need for interventions to make clean, safe water granted, not a privilege.

During the course of the work described in this thesis, several obstacles were met on the way, and to proceed to obtain meaningful, robust results was not always straightforward. Equipment for performing analyses was lacking in the collaborative lab due to the differences in routine analyses performed at the respective laboratories, and had to be transported across the globe, although the method modification made the analytical costs more bearable for WP2. One of the incentives of this thesis that the methods developed here may be used to benefit other projects also aiming to investigate bulk samples at a lower cost, and where the budget may be restrained, the reduction in cost may expand the sample size to give more representative results.

A further challenge was in attempting to ensure that the data generated by my work, which represented just one Work Package (WP2) of a larger project, could be of value in the contexts of the work from other WPs. In particular, the results from WP1, for which data on intestinal parasites in children living in the same areas as sampled in my study, would have

added value to the information that I obtained, as findings regarding genotype, geographical area, sampling season, and factors such as animal ownership or fresh produce consumption, were to be collected and could have been compared with my data. Unfortunately, the partner responsible for WP1 were unable to fulfil their contribution within the timescale of the project. However, sampling has been performed, questionnaires have been completed and analysis and genotyping of these are in progress.

In 2014, India's Prime Minister Modi launched the "Clean India Mission" (Swachh Bharat Abhyian), with the objective of India being a "clean country" by 2019. This mission includes the development of awareness about sanitation, health education and promotion of sustainable sanitation facilities, as well as increasing sanitation coverage and eliminating open defecation by 2019 through the construction of community and public toilets. A staggering 597 million Indians do not have access to toilets, and due to cultural practices, open defecation is preferred in some communities. Between April 2014 and January 2015, 3.2 million toilets have been built, and the Swachh Bharat campaign has made hygiene education a major part of its mission, including regular surveys on toilet use (Jacob, 2016).

Another mission is to eradicate the practice of manual scavenging. The now abandoned caste culture left perceptions that has been suggested to be one of a number of reasons for unclean India, as it externalised the responsibility for maintaining cleanliness to be the duty of a particular caste. Manual scavenging is illegal, but carries on regardless (Jacob, 2016). Some people derive a sense of superiority in littering their environment while others have to clean it, and this perception may be exacerbated in a community where it was expected to be cleaned by the lower-caste scavenger; this is a perception that persists despite the spread of education, globalisation, and urbanisation. Unless the responsibility for cleanliness taken is to the level of each individual, no number of campaigns are going to succeed (Teltumbde 2014), neither in India or any country.

To achieve sustainable changes in an enormous country, it must be realised that cleanliness is more than an adjunct of socioeconomic status, but also a cultural and habitual concern. Countries poorer than India, such as Bangladesh and Sri Lanka, are almost open defecation free (Jacobs, 2016). It is up to each and every individual of any nation, regardless of social status and income, to address these issues, then simple things concerning sanitation and hygiene have the potential to make a huge impact on both population health and the

perception of previously lower castes. Although employment of manual scavengers was prohibited in 1993, over 180 000 households in India are still engaged in manual scavenging (Indian 15th National Census Survey, 2011), indicating that the abolished labour system still prevails.

Another initiative associated with "Swachh Bharat Abhiyan" includes the call for the population to devote 100 hours a year towards the cause of cleaning. The participation and collaboration of all social layers of the population in such initiatives may hopefully give an insight not only to the Indian nation, but all of us, that we are, in fact, one people, all living on the same one globe, all affected by the same One Health.

With studies described in this thesis considered against this background, the following points provide a few concrete suggestions on future research that could build on the results that I obtained and could provide a small contribution towards the "Swachh Bharat Abhiyan" campaign.

- When analysis of the samples obtained in WG1 has been completed, it would be useful to examine the results obtained with those that I obtained from water, vegetables, and different domestic animals. This may indicate the transmission routes and sources of most relevance to the children participating in WG1 as well as indicating which variables are of importance for the possible transmission pathways and any potential foci of infection.
- Clearly fresh produce on sale in Chandigarh is contaminated – but apart from suggesting that customer handling in supermarkets may be one route of contamination, my results are unable to determine the most important routes of contamination. This could be in the field, but other sources that I observed include the buckets of water that are regularly splashed over fresh produce to keep them looking fresh; analysis of these buckets could provide interesting information. Other close investigations of the fresh produce chain, such as harvest, transport and storage may provide other insights and thus offer the opportunity for implementing preventive measures.

- Some of the drinking water available in Chandigarh is contaminated, but, again, how this contamination arises cannot be determined from the information in my studies. Investigation of the barriers for contamination in place for the water supplies could indicate potential weaknesses and indicate where the situation could be improved, for example by catchment control, water treatment initiatives, or efforts to reduce post-treatment contamination that may be associated with leakages and pressure drops within the distribution network.
- Much of the animal sampling was done, by necessity, on an *ad hoc* basis. A more systematic sampling strategy focussed on areas and age groups of particular interest could provide more useful information. My results indicate that backyard goats may be particularly important regarding zoonotic/anthropozoonotic transmission of *Giardia*, and closer investigation of this potential transmission cycle could provide useful insights. In addition to addressing the human-goat-human transmission web, it would also be of value to learn the clinical significance of *Giardia* for the goats themselves; although overt diarrhoea was not observed, more subtle effects, such as reduced weight gain and lower milk production, may mean that *Giardia* in goats not only has the potential to affect the owners by being a reservoir of infection, but also reduce the economic gains that the owners hope to derive from goat husbandry.
- Although the main focus of my research was on the (potentially) zoonotic intestinal protozoa, *Cryptosporidium* and *Giardia*, other zoonotic parasites are also worthy of further exploration. A 17-year time-series study on the occurrence of cysticercosis conducted during this study (Robertson et al. 2017), indicated that this remains an important disease in Chandigarh, particularly in women, despite the very low level of meat consumption in this society. This is due to the enormous potential for environmental contamination from a single individual with taeniosis, and the fact that pigs tend to be kept in the poorest areas of the city where open defecation is a fact of life. Exploration of the prevalence of porcine cysticercosis, the extent of contamination of the environment with *Taenia* eggs, and factors associated with transmission could provide valuable insights that could perform a basis for addressing this issue.

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8. Compilation of papers

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A reduced-cost approach for analyzing fresh produce for contamination with *Cryptosporidium* oocysts and/or *Giardia* cysts

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ABSTRACT

Fresh produce is a recognized vehicle for transmission of various protozoan parasites, including *Toxoplasma gondii*, *Cyclospora cayetanensis*, *Giardia duodenalis*, and *Cryptosporidium* spp. For *Giardia* and *Cryptosporidium*, a Standard ISO Method for analyzing fresh produce is being developed, based on the standard methods for analyzing water. Although it is undoubtedly of value to have a Standard Method available, if the Method is very expensive or difficult to perform this may hamper routine surveys, particularly in settings where resources are restrained, although arguably such settings may produce the results of most importance. Here we present a modified method for analyzing green leafy vegetables such as lettuce or spinach for *Cryptosporidium* oocysts and *Giardia* cysts.

The modified method is considerably cheaper than the Standard Method; by using a smaller volume of magnetic beads in the immunomagnetic separation (IMS) step and buffers that are complementary to those provided in the IMS kit, the cost per analysis is reduced significantly.

In-house seeding trials resulted in acceptable levels of recovery. The modified method has also been trialed in 10 different microbiology analysis labs with experience of detecting protozoa, and results have been shown to be satisfactory; recovery rates ranged from 4% to 88% with a mean of 53% for *Cryptosporidium* and 33% for *Giardia*. Generally poor results were associated with problems in shipping reagents. This modified method is not proposed as an alternative to the Standard Method, but as a complementary approach providing a cheaper option for projects on limited budgets or for laboratories performing analyses in situations or countries where application of the ISO Standard Method is too expensive.

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1. Introduction

Fresh produce is a recognized vehicle for transmission of various protozoan parasites, including *Toxoplasma gondii*, *Cyclospora cayetanensis*, *Giardia duodenalis*, and *Cryptosporidium* spp.

Although such protozoan parasites do not multiply in foodstuffs, they can survive in or on moist foods for months. Such transmission routes can generally only be identified in an outbreak situation in which several people become clinically ill due to consumption of contaminated products. Outbreaks of protozoan foodborne infections are relatively rare compared with bacterial or viral pathogens, though the epidemiology of this route of infection is evolving and outbreaks or single cases of infection may become more numerous in the future (Pollock & Nichols, 2012).

Outbreaks of foodborne protozoan infection in recent years have highlighted the need for development of a method for investigating fresh produce for contamination with the transmission stages.

Standard Methods for analysis of water samples for *Cryptosporidium* oocysts and *Giardia* cysts have been available for several years (e.g. ISO 15553, 2006; US EPA 1623, 2005). Initial work in developing a Standard Method for analyzing fresh produce for contamination has largely focused upon these two parasites, and the methods have been based broadly on the water method, with elution from the surfaces of the fresh produce as the initial steps (Cook et al., 2006a, 2006b, 2007; Robertson & Gjerde, 2000, 2001). In brief, these methods depend on an elution step, followed by concentration based on centrifugation and immunomagnetic separation (IMS), and detection by immunofluorescent antibody testing (IFAT). The method described by Cook et al. (2006a), has been tested in a round-robin interlaboratory trial (Cook et al., 2006b). This method has been used as the basis for the development of an ISO Standard Method (ISO 18744; Microbiology of the food chain — Detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits), which is in the final stages of adjustment and approval before release.

Although it is undoubtedly useful to have an ISO Standard Method, choice of a specific method always raises some concerns. In this case, the concerns include that the recovery efficiencies achieved by Cook et al. (2006a, 2007) were not duplicated in independent or semi-

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independent studies (e.g. Amoros, Alonso, & Cuesta, 2010; Rzezutka et al., 2010); even in the validation studies in which 'expert' labs participated, although recovery efficiencies for *Cryptosporidium* from raspberries were similar to those of the developing lab, the recovery efficiencies of *Cryptosporidium* from lettuce were significantly lower (Cook et al., 2006b) and also misidentification of oocysts was a problem, despite the participating labs being described as 'expert'. Furthermore, the Standard ISO Method 18744 is for both *Cryptosporidium* and *Giardia*, but the publications on which the Standard Method are largely based (Cook et al., 2006a, 2006b), only address *Cryptosporidium parvum*.

A further concern is the cost of the method, which may result in the use of the Standard Method being prohibitively expensive, especially in countries where it may provide the most valuable results. Here we describe experiments to develop a reduced cost version of this method and its validation through independent inter-laboratory trials.

Thus, the intention of this article is not to provide a replacement for ISO Method 18744, which we believe should always be used in situations such as outbreak investigation, but to provide a complementary method that is cheaper and still robust, as demonstrated by testing in a blinded ring-trial of different laboratories. It may be more suitable for use in situations when a more reduced price is important, for example in research projects with limited budgets and for laboratories performing routine analyses in countries where application of the ISO method is too expensive.

2. Materials and methods

2.1. Method development

2.1.1. Fresh produce

Although a range of fresh produce can be contaminated with protozoan parasites, types that are eaten raw are more likely to act as transmission vehicles resulting in infection. These produce include berry fruits (which has been particularly associated with outbreaks of cyclosporiasis) and salad vegetables. The latter has been associated with outbreaks of cryptosporidiosis (Robertson & Chalmers, 2013). Thus, for the purposes of method development, all fresh produce used were green leafy vegetables (iceberg lettuce), purchased locally at a general store and used immediately. For each spiking experiment, 30 g samples of lettuce leaves were used.

2.1.2. Parasites

Initial studies were based on *Cryptosporidium* oocysts and *Giardia* cysts isolated from animal fecal samples submitted to the diagnostic laboratory, purified by sodium chloride flotation, and held refrigerated. Dilutions were made using Kova Glasstic slides. For each sample spiked, three control spikes (directly to a Spot-on slide) were used to determine the size of spike used.

Following the initial studies, testing of the method internally used commercially-obtained spike samples (AccuSpike™-IR; Waterborne Inc., New Orleans, USA and EasySeed™, TCS Biosciences Ltd, Botolph Claydon, UK) in which known numbers of *Cryptosporidium* oocysts and *Giardia* cysts (around 100) have been sorted by flow cytometry into a precise volume of buffer.

2.1.3. Seeding of samples

For sample seeding, the lettuce leaves were pre-weighed into a homogenizer bag with a filter (Seward BA6041/STR filter bag) before the spike was added. The samples were then left to dry for at least 5 h, more often overnight, at ambient temperature.

For initial experiments, parasites were seeded directly into a glycine buffer and also into glycine buffer eluate obtained from washing non-seeded lettuce samples, such that other contaminants from the lettuce samples were in the buffer solution used for seeding experiments.

2.1.4. Elution of parasites from lettuce samples and concentration of eluate by centrifugation

This method is the same as that described in Draft ISO Method 18744. In brief, 200 ml of 1 M glycine was added to the stomacher bag containing the spiked lettuce leaves, and mixed well by hand from outside the bag (external manipulation) to ensure the leaves are covered. The bag containing the leaves and solution was then stomached for 1 min in a paddle beater (stomacher). The eluate was collected into 50 ml centrifuge tubes and then the bag and produce were rinsed thoroughly with 2×20 ml distilled water, which was poured into the 50 ml tubes. The produce and bag were discarded, and the eluate concentrated by centrifugation at 1550 rcf for 10 min. The supernatant was removed from each tube by aspiration, and the pellets resuspended, combined, and re-centrifuged until a single 50 ml tube with a 5 ml concentrate remained. This was resuspended in a weak detergent solution containing SDS (sodium dodecyl sulfate), Tween 80, and antifoam A.

2.1.5. Investigation on reduction in quantity of IMS reagents

In terms of reagents, IMS is the most expensive step of the protocol. There is only one supplier of magnetic beads for both *Cryptosporidium* and *Giardia* and buffers (Dynabeads® GC-Combo, Life Technologies, Thermo Fisher Scientific Inc.) and therefore the option of using alternative beads is excluded. However, as the IMS beads are used in excess in the Standard Method (100 μl of each bead type), it was hypothesized that the method may be equally successful with fewer beads.

The protocol provided by the bead manufacturer is explicitly directed towards the analysis of water sample concentrates, and therefore the different factors in concentrates from fresh produce eluates may lend themselves to manipulating the method so that smaller quantities of reagents are used. Indeed, the publication by Cook et al. (2006a) states that another IMS kit (now no longer commercially available) outperforms the one used, "particularly when higher particulate densities or elevated divalent cation concentrates are encountered". Additionally, the work by Robertson and Gjerde (2001) also suggests that the IMS step could be adjusted and improved for fruit and vegetable analyses, stating "an improved IMS technique thus has the potential to increase recovery efficiency of the parasites". Thus, although both these publications (Cook et al., 2006a; Robertson & Gjerde, 2001) discuss the potential for altering or tweaking the IMS step for analysis of fresh produce, their focus is on improving recovery efficiency, while the focus of the research described here was on using less reagents (and thereby decreasing the cost per analysis), while maintaining an acceptable recovery efficiency.

2.1.5.1. Reduction of volume of beads used in analysis. IMS was attempted on spiked concentrates using 100 μl beads (standard volume), 50 μl beads, 20 μl beads, and 10 μl beads, but with the same buffer volumes as used for each experiment as for when 100 μl of beads are used (i.e. 1 ml SL-Buffer A and 1 ml SL-Buffer B for the initial capture). When initial results had identified a potential reduction in bead volume that continued to provide acceptable recovery efficiencies, then this was investigated more closely in three different replicate experiments using commercially obtained spiked samples, with seeding into either glycine buffer (twice) or glycine buffer eluate (once) with five replicates per seeding experiment.

2.1.5.2. Buffering of samples for IMS analysis. As bead reduction was found to provide acceptable recovery efficiencies, decreasing the buffers commensurately was investigated as the buffers cannot be obtained separately from the supplier. These experiments were conducted in duplicate with seeding of parasites into glycine buffer eluate, with five replicates per seeding experiment.

Results suggested that the buffers are an essential part of the IMS procedure, and reducing the buffer volumes has a negative effect on recovery efficiency. Thus, the IMS buffering must be adjusted in order to maintain recovery efficiency but, at the same time, reduce costs.

PBS-Tween-20 buffers of different concentrations (ten times concentrations of 0.02%, 0.05%, 0.1%, 0.2%, 0.5%) were investigated to supplement SL-buffer A, while a range of commercially available buffers (SurModics) were investigated for supplementing SL-buffer B.

2.2. Final protocol for internal testing and independent inter-laboratory trials

Based on the results from the testing of reduced volumes of beads and buffers, a final protocol was described. Following elution and concentration of the eluate by centrifugation as previously described, the eluate is transferred to a glass L10 tube, along with rinsate from the centrifuge tube, so that the final volume in the L10 tube is 10 ml. Purification/isolation by IMS can, then, follow the standard protocol described for water by the manufacturer. However, 0.05% PBS-Tween 20 is used instead of 10× SL-A buffer of the IMS kit (or 200 μl of 10 × SL-A buffer is used and 800 μl of 0.05% PBS-Tween 20) and a commercially available buffer (SurModics StabilZyme AP buffer, abbreviated to SM-SZ here) is used to replace 800 μl of buffer SL-B. Thus, instead of using 1000 μl SL-B buffer as in Draft ISO Method 18744, 200 μl of buffer SL-B is used along with 800 μl of SM-SZ.

Following mixing between the eluate, beads, and buffers for 1 h, the beads are collected using a magnet, and the eluate, buffers, and any debris were discarded. The beads are then transferred to a smaller tube (1.5 ml centrifuge tube) in a small volume, the beads collected again, and finally the beads dissociated from the parasites by vigorous shaking in 50 μl 1 M hydrochloric acid.

Detection by IFAT using DAPI and Normarski optics are as described in the Draft ISO Method 18744 or in the standard methods for analysis of water concentrates (US EPA 1623, 2005; ISO 15553, 2006).

2.2.1. In-house testing of protocol

The protocol developed was tested in the developing lab by another analyst not involved in the initial developmental work to ensure acceptable in-house recovery efficiencies before initiating an independent round-robin trial of this method. Four replicates and one control of spiked samples of Romaine lettuce were used, with spiking with commercially-obtained spike samples (EasySeed™).

2.2.2. Independent multi-laboratory testing of final protocol

For a more thorough and systematic evaluation of the method, a round-robin test was organized with 10 participating “expert labs”, 8 from within Europe, 1 in Malaysia and 1 in Canada. Each lab that agreed to participate was sent a detailed protocol several weeks before the distribution of samples, including a list of the necessary equipment (see Supplementary material 1). The labs were asked their preferred time for receiving the samples for analysis, then each lab was sent two tubes (labeled A and B) that they were informed were parasites for test spiking, two single-welled spot-on slides, and 5 labeled tubes containing: a) PBS-Tween 20 (labeled Q4), b) buffer SL-B (labeled as such), c) SurModics StabilZyme AP buffer (labeled SM-SZ), d) Dynabeads anti-*Cryptosporidium* (labeled as such), e) Dynabeads anti-*Giardia* (labeled as such). Some labs had requested other reagents or disposables required in the method including anti-foam A and stomacher bags. These were provided in the package as requested.

The two spike tubes (A and B) looked identical. In order to save costs of running this trial, the spikes sent were equivalent to 50% EasySeed™ spikes that had been prepared in-house. This was done by shaking individual EasySeed™ spike vials vigorously and then dividing into two aliquots, each containing 50% of the original spike.

Thus, each participant was sent a seeding sample containing approximately a 50% EasySeed™ size spike and an empty EasySeed tube that had been cleaned by soaking in 15% sodium hypochlorite overnight, then washed in hot soapy water three times, and then rinsed, and contained only water (same volume as for the spike) — that is a negative control. None of the participants were aware that only one sample contained parasites.

A trip control was sent out on two distributions (analyzed in the sending lab), and recovery efficiencies of 54% and 66% for *Cryptosporidium* recorded and 44% and 56% for *Giardia*.

Participants were requested to buy their own leafy green vegetable for spiking, and to record details on the form provided. Should parasites be detected in samples that were not spiked with parasites, these numbers were taken into account in calculating the recovery efficiency in the spiked samples.

2.2.3. Statistics

Descriptive statistics were used in the development of the modified method. For comparison of the results obtained in testing the modified method, the approach described by Langton, Chevennement, Nagelkerke, and Lombard (2002) was used.

For the spiked samples, the accuracy of the method is described by the sensitivity and specificity parameters, that is the percentage of known positive test material that were correctly defined as such (sensitivity) and the percentage of known negative test correctly identified as such (specificity). However, as the vegetable used for spiking onto was not provided, but actually purchased by the individual analytical laboratories, there was a risk for the negatives actually being positive due to low-level contamination. Although the mean spike size for each parasite was found to be 43 for *Cryptosporidium* and 42 for *Giardia* based on replicate counts, the theoretical amounts (50 of each parasite) were used in the calculations in order to be conservative in estimating recovery efficiencies.

In calculating the percentage of false negatives and mean recovery efficiencies, the confounding factors listed by Scotter et al. (2001) were considered for exclusion of results (temperature abuse during shipment; clear deviations from the method in the testing laboratory; questionable laboratory performance). Determinations were performed according to the following equations (Cook et al., 2006b):

$$\%\,\text{false negatives} = \frac{\text{Number of samples where threshold level not met}}{\text{Number of samples to which parasites were added}} \times 100$$

$$\%\,\text{false positives} = \frac{\text{Number of samples to which parasites were not added, but parasites were detected}}{\text{Number of samples to which parasites were not added}} \times 100.$$

3. Results

3.1. Reduction in volume of beads used for IMS step

For initial studies with seeding onto lettuce, an acceptable recovery efficiency was considered to be 30%. Recovery efficiencies from spiking into glycine will be higher as the elution and concentration steps are excluded. The lowest volume of beads found to give an acceptable recovery rate was 20 μl (Tables 1 and 2).

Table 1
Initial recovery efficiencies from lettuce samples seeded with *Cryptosporidium* oocysts and *Giardia* cysts using different volumes of IMS beads, but non-adjusted volumes of buffers (same volume of buffers used as for 100 μl beads).

	Mean % recovery efficiency (n)	
	Cryptosporidium	*Giardia*
100 μl beads	67 (n = 5)	58 (n = 5)
50 μl beads	49 (n = 5)	61 (n = 5)
20 μl beads	30 (n = 5)	45 (n = 5)
10 μl beads	18 (n = 5)	26 (n = 5)

Table 2
Comparison of recovery efficiencies from glycine seeded with *Cryptosporidium* oocysts and *Giardia* cysts using either 100 μl or 20 μl of IMS beads, but standard buffer volumes (as for 100 μl beads).

	Mean % recovery efficiency (n)	
	Cryptosporidium	*Giardia*
Parasites seeded into glycine buffer		
100 μl beads, 1 ml buffers (standard)	57 (n = 5)	71 (n = 5)
20 μl beads, 1 ml buffers	52 (n = 5)	85 (n = 5)
Parasites seeded into glycine buffer		
100 μl beads, 1 ml buffers (standard)	63 (n = 5)	73 (n = 5)
20 μl beads, 1 ml buffers	62 (n = 5)	95 (n = 5)
Parasites seeded into glycine buffer eluate — (obtained from non-seeded lettuce)		
100 μl beads, 1 ml buffers (standard)	60 (n = 5)	82 (n = 5)
20 μl beads, 1 ml buffers	59 (n = 5)	78 (n = 5)

3.2. Buffering of samples for IMS analysis

Initial experiments found that if the volume of buffers was also decreased commensurate with the volume of beads, then recovery efficiency was reduced (Table 3). This indicates the importance of the buffers for recovery of the parasites, and that if the volume of beads is to be reduced, then the buffering must also be adjusted in order to obtain acceptable recovery efficiency at a reduced price.

A range of buffers were tested as replacements or supplements for the buffers provided by the IMS kit (Table 4).

Two buffers were identified that could be used in conjunction with a lower volume of kit buffers and provided equivalent results to those obtained when the standard IMS procedure was used. The buffer found to provide a satisfactory replacement for buffer SL-A provided in the IMS kit was 0.05% PBS-tween buffer. This could either entirely replace buffer SL-A or a mixture could be used, as long as a final buffer volume of 1 ml was added to the 10 ml sample eluate concentrate for analysis.

The second buffer found to provide a satisfactory complement to buffer SL-B in the IMS kit was commercially available buffer (SurModics StabilZyme® AP buffer, described in the tables here as SM-SZ).

3.3. In-house testing of final protocol internally

In-house testing of the final protocol with four replicate spike samples and analysis conducted by an analyst who had not performed the initial method development experiments demonstrated that a recovery efficiency of approximately 50% could be expected for the whole analysis using the protocol described (Table 5).

Table 3
Comparison of recovery efficiencies from glycine seeded with *Cryptosporidium* oocysts and *Giardia* cysts using either 20 μl of IMS beads and either 1 ml (standard) or 200 μl buffer volumes.

	Mean % recovery efficiency (n)	
	Cryptosporidium	*Giardia*
Parasites seeded into glycine buffer eluate — (obtained from non-seeded lettuce)		
100 μl beads, 1 ml buffers (standard)	75 (n = 5)	89 (n = 5)
20 μl beads, 1 ml buffers	75 (n = 5)	82 (n = 5)
20 μl beads, 200 μl buffers	36 (n = 5)	52 (n = 5)
Parasites seeded into glycine buffer eluate — (obtained from non-seeded lettuce)		
100 μl beads, 1 ml buffers (standard)	98 (n = 5)	71 (n = 5)
20 μl beads, 200 μl SL-A, 1 ml SL-B	57 (n = 5)	57 (n = 5)

Table 4
Comparison of recovery efficiencies from glycine seeded with *Cryptosporidium* oocysts and *Giardia* cysts using either 20 μl of IMS beads and different buffers.

	Mean % recovery efficiency (n)	
	Cryptosporidium	*Giardia*
Parasites seeded into glycine buffer eluate — (obtained from non-seeded lettuce)		
1 ml SL-A, 1 ml SL-B (standard)	86 (n = 2)	76 (n = 2)
1 ml 0.1% PBS-Tween, 1 ml SL-B	36 (n = 2)	15 (n = 2)
1 ml 0.2% PBS-Tween, 1 ml SL-B	43 (n = 2)	34 (n = 2)
1 ml 0.05% PBS-Tween, 1 ml SL-B	84 (n = 2)	83 (n = 2)
Parasites seeded into glycine buffer eluate — (obtained from non-seeded lettuce)		
1 ml 0.05% PBS-Tween, 1 ml SL-B	58 (n = 3)	56 (n = 3)
200 μl 0.05% PBS-Tween, 800 μl SL-A, 1 ml SL-B	64 (n = 3)	55 (n = 3)
1 ml 0.05% PBS-Tween, 200 μl SL-B, 800 μl SM-SZ	83 (n = 3)	59 (n = 3)
1 ml 0.05% PBS-Tween, 1 ml SM	55 (n = 3)	77 (n = 3)
Parasites seeded onto 30 g lettuce		
1 ml SLA, 1 ml SL-B (standard)	36 (n = 2)	31 (n = 2)
1 ml 0.05% PBS-Tween, 1 ml SM	44 (n = 2)	55 (n = 2)
1 ml Q4, 200 μl SL-B, 800 μl SM-SZ	45 (n = 2)	60 (n = 2)
Parasites seeded onto 30 g lettuce		
1 ml SLA, 1 ml SL-B (standard)	36 (n = 2)	28 (n = 2)
1 ml 0.05% PBS-Tween, 200 μl SL-B, 800 μl SM-SZ	35 (n = 2)	34 (n = 2)
Parasites seeded onto 30 g lettuce		
1 ml SLA, 1 ml SL-B (standard)	37 (n = 2)	33 (n = 2)
1 ml 0.05% PBS-Tween, 200 μl SL-B, 800 μl SM-SZ	45 (n = 2)	51 (n = 2)

3.4. Independent inter-laboratory testing of final protocol

3.4.1. Verification of contamination level of distributed (oo)cysts

Due to resources for this study being limited, it was not possible for each participant to be sent an EasySeed™ vial. Thus, EasySeed vials were divided into two to give a theoretical mean of 50 of each parasite in each vial. Internal controls counting divided samples demonstrated that for two vials (four counts), the mean spike for *Cryptosporidium* was 43 oocysts, standard deviation (SD) of 4.24 for *Cryptosporidium* and for *Giardia* the mean spike size was 42 cysts with SD of 1.9.

3.4.2. Lab characteristics and results

The first samples were distributed to the participating labs in September 2014. Unfortunately, although delivery within 2–3 days was assured by the carrier, the delivery that occurred the most quickly (to Belgium) took 1 day, while for samples sent to Spain, Canada and UK, over 10 days and could have impacted on results, especially for laboratories where the ambient temperature was over 25 °C. For some laboratories, a repeat distribution of samples using a different delivery service was organized, and mostly resulted in more rapid delivery for most participants and improved recovery efficiency. However, for

Table 5
Results from in-house testing of final protocol by a second analyst.

	No. parasites recovered (Confidence interval)	
	Cryptosporidium	*Giardia*
Estimated seed (EasySeed™ size spike)	98 ± 2.1	100 ± 2.4
Negative control	0	0
Trial 1	48	55
	(47.9–50.1)	52.6–57.4
Trial 2	51	63
	49.9–54.1	60.6–65.4
Trial 3	43	49
	41.8–46.0	46.6–51.4
Trial 4	54	56
	53.0–57.2	53.6–58.4

participants in more distant labs where delivery still took over 7 days and were exposed to high temperature, results remained poor.

Delivery times, other deviations, lab-specific details of the trials, and results for detection of the *Cryptosporidium* and *Giardia* seeded onto lettuce are presented in Table 6.

Considerable inter-lab variability in the recovery efficiency of the method was seen, and if all data are included (apart from the trip-control data from the sending lab) but including all other data, then the mean recovery efficiency (±SD) for *Cryptosporidium* is 35 (±30) and for *Giardia* is 18 (±20), with 13 data points for each parasite included in the calculations. However, if the confounding factors mentioned by Scotter et al. (2001) are used for exclusion of some results (temperature abuse during shipment; clear deviations from the method in the testing laboratory; questionable laboratory performance), then the results are much higher. Thus, removing the data from lab 3 trial 1, lab 4 trial 1, lab 5, lab 6, lab 7 trial 1, and lab 9 based on these criteria, then only 7 data points are used for each parasite in the calculations, and the mean recovery efficiency (±SD) for *Cryptosporidium* is 53 (±28) and for *Giardia* is 33 (±22). As the SD remains high, these data as means are not particularly useful, but do indicate that the Method can be implemented successfully in different laboratories. If the trip control data are also included (two more data points per parasite), then the mean recovery efficiency (± SD) for *Cryptosporidium* is 54 (±25) and for *Giardia* is 37 (±21). If a cut-off level of 20% recovery efficiency is set as being acceptable for each parasite, then of the 7 round robin laboratories whose data are included according to the criteria of Scotter et al. (2001), 6 were able to achieve acceptable recovery efficiencies on at least one occasion. The one laboratory (laboratory 3) that did not had prolonged transport experiences, with shipping taking 8 days even on the second sending.

3.4.3. Sensitivity, specificity, accordance and concordance

The sensitivity (samples correctly identified as positives) for the detection of *Cryptosporidium* and *Giardia* in the collaborative trial was 87.5% and 75%, respectively. The percentage of false positives for *Cryptosporidium* and *Giardia* was 0% and 12.5%, respectively.

The specificities (percentage of samples correctly identified as negatives) for the detection of *Cryptosporidium* and *Giardia* in the collaborative trial were 87.5% and 100%, respectively. False negatives for *Cryptosporidium* and *Giardia* were 12.5% and 25%, respectively; this is not necessarily true false, but not reaching a threshold of 20% recovery.

The concordance was, for the trial in total, 80% (Langton et al., 2002).

Accordance could only be calculated from the developing lab, as this is the only lab where multiple trials were conducted, and is 100%.

4. Discussion

In this article we present a modified method for analyzing fresh produce for contamination with *Cryptosporidium* and *Giardia*. The method is based on previous publications and also on the Draft ISO Method 18744. However, by reducing and adapting the IMS stage of the protocol, by using only 20% of the amount of beads, and providing a modified buffering system, the cost of the analysis is also reduced considerably. In-house recovery efficiencies of around 50% were obtained for each parasite using this modified method, which is equivalent to that which may be expected using other published methods or the Draft ISO Method 18744 that has yet to be approved.

In a previous inter-laboratory validation exercise somewhat similar to this one, and on the results of which Draft ISO Method 18744 is based, and that was concerned with the detection of contamination of

Table 6
Round-robin testing of final protocol in independent labs: lab-specific characteristics and results obtained.

Lab no.	Lab location	Trial no.	Period between dispatch and delivery of spikes & reagents (days)	Lettuce used (weight)	Deviations from protocol (including detection of parasites in non-spiked sample)	Percentage recovery efficiency (based on theoretical spike of 50 parasites)	
						Cryptosporidium	*Giardia*
1	Oslo (trip control)	1	2	Iceberg (30 g)	None	54	44
1	Oslo (trip control)	2	1	Iceberg (30 g)	None	66	56
2	Belgium	1	1	Green lettuce (30 g)	None	40	26
3	Canada	1	17	Iceberg (30 g)	None	0	0
3	Canada	2	8	Iceberg (30 g)	None	12	6
4	Denmark	1	7	Iceberg (30 g)	Pulsifier used instead of paddle beater; 2 oocysts and 1 cyst detected in non-spiked sample	20	0
4	Denmark	2	1	Iceberg (30 g)	None	30	68
5	Finland	1	7	Cabbage (30 g)	Hand manipulation instead of stomacher, but for 2 min instead of 4 min. Detergent step not included	28	4
6	Malaysia	1	6	Lettuce (48 g)	Exposure to prolonged high temperature during transport reported. 48 g of leaved vegetable used (protocol states 30 g)	0	0
7	Poland	1	8	Butterhead lettuce (30 g)	None	30	4
7	Poland	2	1	Butterhead lettuce (30 g)	None	84	20
8	Spain	1	7	Curly lettuce (30 g)	5 oocysts detected in non-spiked sample	50	56
9	Spain	1	7	Romaine lettuce (30 g)	Exposure to prolonged high temperature during transport reported	8	4
10	Sweden	1	8	Romaine lettuce (30 g)	None	64	32
11	UK	1	10	Iceberg lettuce (30 g)	None	88	22

fresh produce with only *Cryptosporidium* oocysts (Cook et al., 2006b), the already spiked samples were distributed to the expert labs (8 labs, all in UK). Three levels of spike were used and also negative controls. In our exercise, only 1 positive spike was used and 1 negative control, and the participants provided their own leafy produce for spiking. However, despite the possibility of some participants using a naturally contaminated product for spiking, only two labs detected parasites in the negative control sample. Given the low level of parasites tested in this case it seems possible that this was natural contamination rather than cross-contamination in the laboratory.

In the exercise by Cook et al. (2006b), five out of eight labs detected *Cryptosporidium* oocysts on samples that were known to be negative, and thus these results compare favorably.

In our study, problems with delivery of the samples (with delivery taking up to 17 days, and at temperatures of around 28 °C, with the cold block in the package entirely defrosted by delivery) are considered to have resulted in probable inactivation of the Dynabeads (or the activity of the antibody on the Dynabeads), and hence very low or negative samples from positive spikes. It seems unlikely that other reagents in the package would have been so adversely affected, although some parasites may have become deformed. Sending repeat samples with a different delivery service resulted in more rapid delivery for most participants and improved recovery efficiency. However, for participants in more distant labs where delivery still took over 7 days and were exposed to high temperature, recovery efficiency remained very low. In the exercise by Cook et al. (2006b), in which delivery presumably occurred within a matter of days, one lab failed to detect oocysts at a spike level of between 50 and 100 oocysts. This demonstrates that variation does occur in such exercises, and also that shipment is critical. This is a well-recognized weak spot for inter-laboratory trials.

The fact that some shipments took longer time than anticipated, and that some labs thus received a thawed cooling block with temperate reagents justifies the exclusion of some of the results from this trial. Other labs who also received their reagents over the proposed time managed to obtain acceptable recoveries, though these labs were situated in more temperate regions and it is plausible to assume that the temperature exposed to the parcel during the prolonged transport inactivated the beads. In addition, some labs requested a new set of reagents after experiencing poor recoveries in the initial trial, and significantly improved their results with reagents shipped more swiftly.

The exclusion of results was based on criteria set by Scotter et al. (2001):

1. Test material had received a significant temperature abuse during shipment.
2. Testing laboratory had clearly deviated from the specified standard operating procedure.
3. Performance of laboratory was questionable as indicated by large numbers of false-positive or false-negative results more than would be expected by chance.

Though these criteria were originally set for the detection of bacteria in food, they can be applied to our study also as the same variables are of relevance (temperature, method deviation and detection of false-positives).

Although the results of the inter-laboratory trial did not provide as high or stable recovery efficiencies as were found in the developing lab, this is probably to be expected given that the developing lab had used considerable time on the method and therefore was more experienced. Furthermore, shipping problems (delays and exposure to elevated temperatures) apparently affected the recovery efficiencies. It is worth noting that although all the laboratories that participated in the trial are considered to have expertise in the analysis of samples for these parasites, method-specific training was not provided. This suggests that the modified method is sufficiently simple and robust that it can be readily implemented into a competent laboratory without difficulty.

5. Conclusions

Based on the results of these experiments, we believe that the modified method developed and tested here provides a useful complementary approach to the Draft ISO Method 18744, particularly useful for research projects with limited funding or for use in situations where resources are stretched. The next step for validating this method will be to return parallel analyses on naturally contaminated fresh produce.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2015.05.010.

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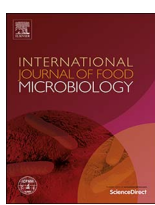

Keeping it cool: Survival of *Giardia* cysts and *Cryptosporidium* oocysts on lettuce leaves

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ABSTRACT

Fresh produce has been recognized as a vehicle for transmission of protozoan parasites for many years, and there are numerous publications regarding their occurrence on such foodstuffs, indicating their potential importance as foodborne parasites. Nevertheless, few studies have been published regarding the effectiveness of this transmission route, and whether contamination is likely to result in transmission. The purpose of this study was to assess the viability of *Cryptosporidium* oocysts and *Giardia* cysts, two protozoa associated with both waterborne and foodborne transmission, by spiking fresh produce (lettuce leaves) with viable transmission stages and determining changes in viability. These investigations were performed under different conditions and over time spans that may be used in a regular household; a fridge at 4 °C, under ambient temperatures exposed to natural cycles of light during night and day, and inside a cupboard to ensure no light exposure, for a duration of up to two weeks, or as long as the produce remained visually palatable. The major finding from this study is that whereas both *Cryptosporidium* oocysts and *Giardia* cysts survive well when kept moist and refrigerated, survival of *Giardia* cysts was abrogated on lettuce at room temperature. Indeed, almost 50% die-off of *Giardia* cysts was recorded within the first 24 h.

Cryptosporidium oocysts had a stable viability throughout the experiment under all the conditions investigated, indicating that fresh produce is a suitable transmission vehicle for *Cryptosporidium*, even if contamination occurs on-farm and the parasites are exposed to non-favourable storage conditions, as may be common in developing countries.

Giardia cysts were not as robust as *Cryptosporidium* oocysts, and would be probably unlikely to survive under ambient storage conditions on-farm, during sale, or at home. However, if kept refrigerated, then some contaminating *Giardia* cysts may remain viable and therefore may pose a threat to the consumer.

Thus, as the cold chain for transport and storage of fresh produce improves, it is important that similar improvements are implemented to reduce the contamination of fresh produce with parasite transmission stages.

1. Introduction

The protozoan parasites, *Cryptosporidium* spp. and *Giardia duodenalis* are among the most frequently found intestinal protozoan parasites in humans worldwide. Both parasites can cause diarrhoeal disease, and a global study revealed that *Cryptosporidium* spp. and *G. duodenalis* are two of the most common aetiological agents in paediatric diarrhoea in developing countries, and are associated with mortality as well as morbidity (Kotloff et al., 2013). Also, 8–19% of diarrhoeal diseases can be attributed to *Cryptosporidium* in developing countries (Gatei et al., 2006), and 10% of the population in developing countries excretes oocysts. In developed countries, this proportion is estimated to be 1–3% (Lozano et al., 2012). For *G. duodenalis*, an estimated 280 million cases occur annually (Lane and Lloyd, 2002). Both parasites have long been recognized as being potentially waterborne pathogens, and many outbreaks of waterborne cryptosporidiosis and waterborne giardiasis have been described. Out of 199 reported outbreaks of human disease due to waterborne transmission between 2004 and 2010, *Cryptosporidium* was the aetiological agent for around 60%, and *Giardia* was the aetiological agent for around 35% (Baldursson and Karanis, 2011).

Food, particularly fresh produce eaten raw, has also been recognized as a potential transmission vehicle for these parasites. Although washing fresh produce may reduce the risk of contaminated food being ingested, numerous outbreaks demonstrate that washing is not always effective. Contamination of food has been considered to occur either directly from food-handlers, perhaps infected themselves or in close contact with an infected person or animal, or from contact with a contaminated environment. Such environmental contamination can be

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from soil, particularly soil amended with faeces or manure, or from water such as irrigation water or wash water along the food chain (Cook and Lim, 2012).

As with waterborne cryptosporidiosis and giardiasis, the potential for foodborne transmission is considered rather similar for both parasites. Indeed, one recent estimate regarding the burden of foodborne disease (Hald et al., 2016), concluded that *Cryptosporidium* and *Giardia* were quite similar to each other regarding source attribution. Kirk et al. (2015) used expert elicitation to estimate that whereas around 15% of giardiasis cases were foodborne (uncertainty intervals (UI) of 0.08–0.27), a slightly lower proportion (13%) of cryptosporidiosis cases were foodborne (UI of 0.07–0.24).

Although these data are based on all cases, not just outbreaks, the published outbreak data tend to indicate a different pattern. According to a review article published in 2013 on foodborne cryptosporidiosis, there have been at least 18 outbreaks of cryptosporidiosis in which foodborne transmission has been epidemiologically implicated (Robertson and Chalmers, 2013). At least two more foodborne outbreaks (one in Finland and one in UK) have been published subsequently and are not included in that review (Åberg et al., 2015; McKerr et al., 2012), and 8 of these outbreaks were directly linked to consumption of fresh produce. In contrast, only 9 outbreaks of giardiasis with foodborne transmission proven or implicated have been documented, and of these it may be questionable whether some of these outbreaks were really due to the apparently implicated food (Cook and Lim, 2012).

The reason for this discrepancy between the perceived importance of *Giardia* as a foodborne pathogen and the number of reported foodborne outbreaks, as compared with *Cryptosporidium,* is of interest. Indeed, the same reasons that *Cryptosporidium* and *Giardia* are both suited to waterborne transmission have been cited as to why they are also suited to transmission by foods: they are shed in high concentrations from infected hosts, the infectious dose is low, and they are robust, surviving a number of different environmental pressures. However, whether these factors are of the same importance for foodborne transmission as waterborne transmission may be less clear, and other factors may be of relevance in either transmission or recognition of transmission. For example, the more zoonotic nature of some species of *Cryptosporidium,* such as *C. parvum*, that enables direct transmission from animals, may mean that the relative proportion of foodborne infections is lower in comparison with other routes, such as from animals or via contaminated water (Hald et al., 2016). Alternatively, it is possible that the more acute symptoms of cryptosporidiosis compared with giardiasis may give rise to more rapid diagnosis, and thus the possibility of proper investigation and source attribution.

A third possibility is that food is, indeed, a less efficient transmission vehicle for *Giardia* than for *Cryptosporidium*, and, therefore, despite experts considering that food and water are similar vehicles for both parasites, this is not the case. It is possible that *Giardia* cysts may have less opportunity for contamination of fresh produce, that they may be more easily removed from contaminated produce by food preparation washing procedures, or that the survival of *Giardia* cysts on food matrices may be relatively lower than that of *Cryptosporidium* oocysts. Data on these possibilities are lacking in the literature.

The objective of this study was to investigate the last possibility, and determine whether the relative survival of *Giardia* cysts and *Cryptosporidium* oocysts as contaminants of fresh produce is similar or not.

2. Materials and methods

2.1. Giardia cysts and Cryptosporidium oocysts

Giardia duodenalis cysts, H3 isolate belonging to Assemblage B, were obtained from a commercial supplier (Waterborne Inc., New Orleans, USA) and their initial viability was determined shortly after arrival and prior to contamination by the method described in Section 2.3.

Cryptosporidium parvum oocysts, with species identification by Hønsvall and Robertson (2017) were isolated from faeces of naturally infected calves by salt flotation. For one sample with very high fat content, ethyl acetate sedimentation was used prior to salt flotation, and, as considerable fat quantities remained after flotation, was further purified by immunomagnetic separation (IMS) (Dynabeads® GC-Combo, Applied Biosystems™).

To determine the concentration of (oo)cysts, 10 μl of diluted stock was pipetted on a multispot microscope slide (C.A. Hendley (Essex) LTD), stained with a monoclonal antibody (mAb; Aqua-glo, Waterborne Inc., New Orleans, USA) before enumeration and further dilution.

After purification, and immediately prior to the experiments, the viability of the oocysts was determined by the method described in Section 2.3.

2.2. Food matrix used for survival experiments

Iceberg lettuce was used in these experiments as a representative of a food matrix commonly eaten raw. The lettuce was purchased from a greengrocer and prior to the experiments, 3 leaves were taken from both internal and external layers of the lettuce, weighed, and the intact leaves were analysed for contamination with *Giardia* cysts and/or *Cryptosporidium* oocysts using a previously published and independently validated protocol (Utaaker et al., 2015). None of the samples were found to be contaminated.

2.3. Assessment of parasite viability

Both prior to the experiments, and throughout the experiments, the viability was assessed based on morphology and inclusion and exclusion of the vital dyes 4′,6-diamino-2-phenylindole (DAPI) and propidium iodide (PI). For *Cryptosporidium*, the protocol for staining was as described by Campbell et al. (1992), and a similar protocol was used for *Giardia* cysts. In order to identify the parasites eluted from the lettuce, a monoclonal antibody (mAb; Aqua-glo, Waterborne Inc., New Orleans, USA) was added to the suspension in the final 15 min of the staining procedure. The stained sediment was examined in suspension (with the cover slip on the microscope sealed with nail varnish to avoid drying) by fluorescence microscopy using a Leica DMCB microscope equipped with a UV filter block (350-nm excitation, 450-nm emission) for DAPI and a green filter block (500-nm excitation, 630-nm emission) for PI. Nomarski (differential interference contrast) optics on the same microscope was used to examine morphology of individual cysts and oocysts.

The cysts and oocysts were evaluated and categorised according to exclusion or inclusion of the different stains and their morphology (Campbell et al., 1992). Empty or shrunken (ghost) cysts and oocysts were identified under Nomarski optics, containing no nuclei or shrunken residues thereof. They were also non-refractile, apart from the residual body when present.

PI + cysts and oocysts fluoresce bright red under the green filter block; this fluorescence varies from distinct points corresponding to the sporozoite/trophozoite nuclei, to a more diffuse fluorescence within the cyst or oocyst. Cysts and oocysts were categorised as DAPI +, PI − if they did not include PI (as described above), but the nuclei of the sporozoites/trophozoites fluoresced a distinctive sky blue under the UV filter block. Cysts and oocysts that were neither PI +, nor "ghosts", and which showed either rim fluorescence or absence thereof under UV filter block were considered DAPI −, PI −. The interpretation of these various appearances are described in Table 1. For the purposes of this study, DAPI +, PI − parasites and DAPI −, PI − parasites were summed together as viable or potentially viable.

Table 1
Categorisation of cysts and oocysts according to inclusion and exclusion of vital dyes, DAPI and PI, and morphological parameters.

Cyst/oocyst categorisation	Vital dye inclusion		Nomarski microscopy	Viability designation
	DAPI	PI		
Ghost	No	No	Shrunken, deformed, empty shell, lacking contents	Dead
PI +	Yes	Yes	May be deformed, contents	Dead
DAPI +, PI −	Yes	No	Good morphology, contents	Viable at assay
DAPI −, PI −	No	No	Good morphology, contents	Viable, but may need further trigger to excyst

2.4. Experimental design

Lettuce leaves were removed from both external and internal layers (30 g) and put, intact, directly into food storage containers, in replicates of 4 for each time interval and storage location. Then, the leaves were sprinkled with approximately 50,000 *Giardia* cysts and 50,000 *Cryptosporidium* oocysts. The (oo)cysts, diluted in distilled water from stock solution to a volume of 100 μl, were spread on the lettuce leaves in aliquots of 20 μl using a pipette. The food storage containers were then closed using airtight lids.

The containers containing contaminated leaves were exposed to three different conditions. These were: Condition A (refrigerator at 4 °C), Condition B (laboratory benchtop at ambient conditions and temperatures of around 18 °C, with exposure to light-dark cycles), and Condition C (laboratory cupboard with ambient conditions and temperatures as for Condition B, but with continuous darkness except during sampling).

After different time intervals (between 1 h to 14 days), the leaves or sub-samples thereof were placed into stomacher bags, and the parasites eluted from them according to the method of Utaaker et al. (2015). Shorter time intervals for viability assessment were chosen for *Giardia* cysts, as they are generally considered to be less robust than *Cryptosporidium* oocysts. The eluate was concentrated by centrifugation and the pellets transferred into a microcentrifuge tube. The viability of the parasites isolated from the leaves was assessed as described in Section 2.3.

In general, lettuce leaves that became mouldy or otherwise not palatable during storage inside the container were excluded from the study, such that results included in the analyses were only obtained from leaves with a fresh and crispy texture that are thus likely to be considered suitable for consumption. However, for one sample kept at room temperature and contaminated with *Giardia* cysts, the lid of the food container was, inadvertently, not completely sealed. Although the leaves used for these samples withered within 20 h (and so would be unlikely to be eaten by the consumer in the household setting), the *Giardia* cysts were nevertheless collected and their viability assessed.

In order to determine whether temperature alone, or humidity also, played a role in the survival of the parasite transmission stages, for each condition in which the parasites were spiked onto lettuce leaves a control was also established consisting of the parasites in Eppendorf tubes of 1.5 ml tap water. The viability of the parasites was determined by concentrating the parasites by centrifugation and then using the assay as described in Section 2.3.

For each viability assessment for both lettuce and water, four replicate experimental set-ups were analysed, but as some of the samples withered during the study, results were only obtained for two set-ups at some locations and time points.

2.5. Data handling and statistics

The relative viability at each time point is obtained by normalising the data to the initial viability as described in the following equation (AWWA, 1988; Sattar, 1999):

$$\text{Percentage viability} = (N_t/N_0) \times 100$$

where N_t is the number of viable parasites at time t (of 100 parasites), and N_0 is the number of viable parasites at time 0 (of 100 parasites).

After establishing the database in Excel®, the data were transferred to Stata/SE/14 for Windows, StataCorp, College Station, TX for statistical analyses. Survival was analysed using linear regression model using method as a categorical variable and time (log 10 h) as a continuous predictor was utilised, and a follow up logistic regression comparing the viability data of the parasites on the lettuce from initial contamination point until final sampling point. Standard graphical methods were used to assess mode fits and residual patterns.

Table 2
Giardia cyst viability at different storage locations, with the crude observations from initial viability, and normalised according to a 80% initial viability of cysts.

Time of exposure	Viable cysts on lettuce kept on benchtop		Viable cysts on lettuce kept in fridge		Viable cysts on lettuce kept in cupboard	
	From initial viability (%)	Normalised viability (%)	From initial viability (%)	Normalised viability (%)	From initial viability (%)	Normalised viability (%)
1 h	72	90	73	91	74	93
4 h	76	95	77	96	ND	ND
18 h	ND	ND	52	65	ND	ND
24 h	41	51	78	98	24	30
48 h (2 days)	21	27	78	98	59	74
72 h (3 days)	ND	ND	ND	ND	31	39
96 h (4 days)	30	37	ND	ND	33	41
120 h (5 days)	14	18	38	47	10	13
144 h (6 days)	4	5	14	18	4	5
192 h (8 days)	13	16	53	66	ND	ND
216 h (9 days)	ND	ND	44	55	ND	ND

ND = No data.

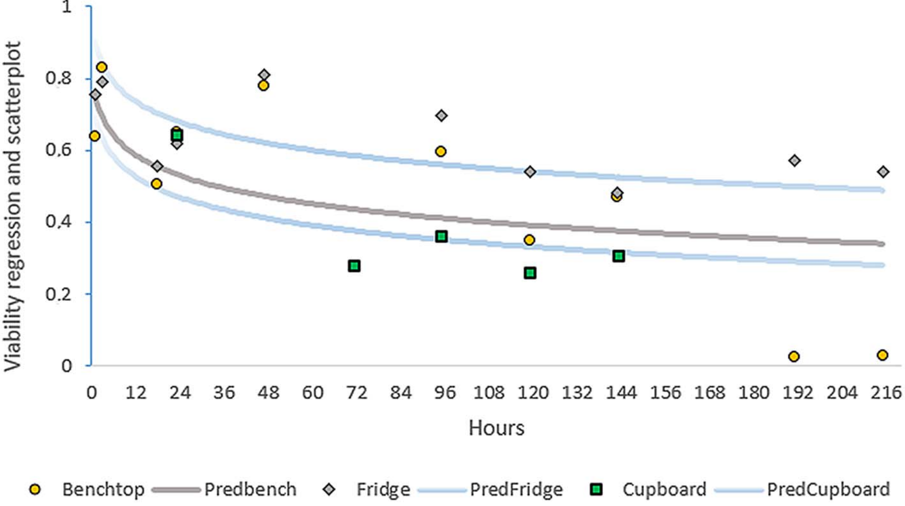

Fig. 1. Rate of decrease in the viability of *Giardia* cysts in lettuce at different storage locations.

3. Results

3.1. Viability of Giardia cysts

The *Giardia* cysts had an initial viability of 80%. The observed viabilities of *Giardia* on lettuce at different sampling times are described in Table 2. After day 6, the lettuce in the cupboard had started to become mouldy and the viability could not be estimated due to debris in the samples occluding the cysts in the microscope. Cysts in the fridge retained their viability longer, and the lettuce remained fresh. On day 9, the viability count for lettuce kept in the fridge was 44%, with a drop from the initial value by around 30%. The linear regression model gave an R^2 (coefficient of determination) of the regression at 0.46. Fig. 1 shows the raw data with regression lines for bench, fridge and cupboard. The viability of cysts stored in the cupboard did not differ from that of those on the bench ($p = 0.99$), whereas the viability was higher for cysts stored in the fridge ($p = 0.008$).

The rates of decrease in the viability of *Giardia* cysts in water are illustrated in Fig. 2. The R^2 of this model was 0.70. Only a major difference was found between benchtop and cupboard ($p = 0.52$), while cysts had a higher viability in the fridge ($p = 0.05$).

3.2. Viability of Cryptosporidium oocysts

The *Cryptosporidium* oocysts had an initial viability of 30%. The reduction in the viability of *Cryptosporidium* oocysts occurred more slowly than for *Giardia* cysts, and, at some locations, there was no change in viability between the initial and final assessments. Viable *Cryptosporidium* oocysts could still be identified on lettuce stored in the fridge for up to 14 days, and the lettuce still appeared to be palatable (Table 3). The rates of decrease in the viability of *Cryptosporidium* oocysts on lettuce are illustrated in Fig. 3. The R^2 of the regression model was 0.28 and only a marginal effect of time was found ($p = 0.13$). As for *Giardia*, there was no difference between bench and cupboard ($p = 0.47$) while viability was higher in the fridge ($p = 0.05$).

The rates of decrease in the viability of *Cryptosporidium* oocysts in water are illustrated in Fig. 4. The R^2 value of the regression was 0.46, and the effect of time on viability was marginal ($p = 0.20$). Viability of oocysts stored on the bench did not differ from the viability of the oocysts stored in the cupboard ($p = 0.13$), whereas oocysts stored in the fridge had a higher viability ($p = 0.002$).

3.3. Effect of desiccation on Giardia cyst viability

One lettuce sample contaminated with *Giardia* cysts was held in a

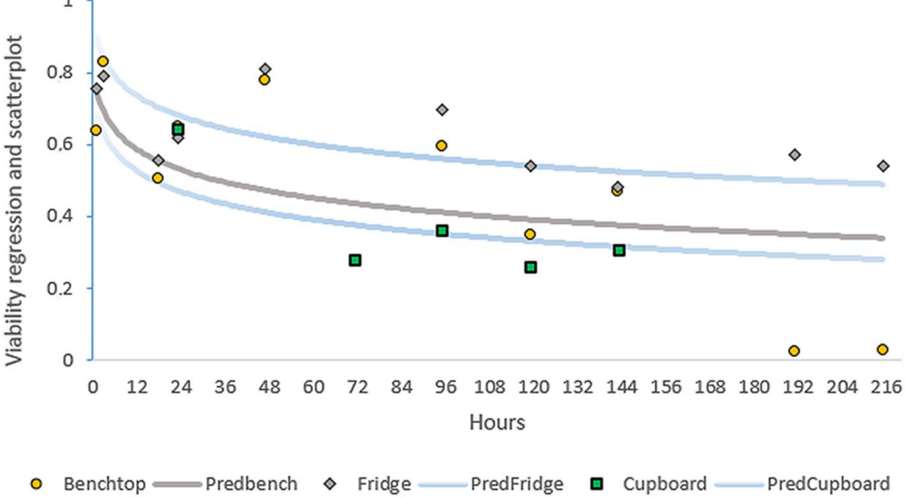

Fig. 2. Rate of decrease in the viability of *Giardia* cysts in water at different storage locations.

Table 3
Cryptosporidium oocyst viability at different storage locations, with the crude observations from initial viability, and normalised according to a 30% initial viability of oocysts.

Time of exposure	Viable oocysts on lettuce kept on benchtop		Viable oocysts on lettuce kept in fridge		Viable oocysts on lettuce kept in cupboard	
	From initial viability (%)	Normalised viability (%)	From initial viability (%)	Normalised viability (%)	From initial viability (%)	Normalised viability (%)
1 day	20	67	35	117	31	102
2 days	10	33	38	125	24	80
3 days	26	87	18	61	17	56
4 days	12	40	29	97	ND	ND
5 days	15	50	16	53	23	77
6 days	29	97	18	61	17	56
9 days	18	60	24	80	ND	ND
14 days	ND	ND	25	83	ND	ND

ND = Not done.

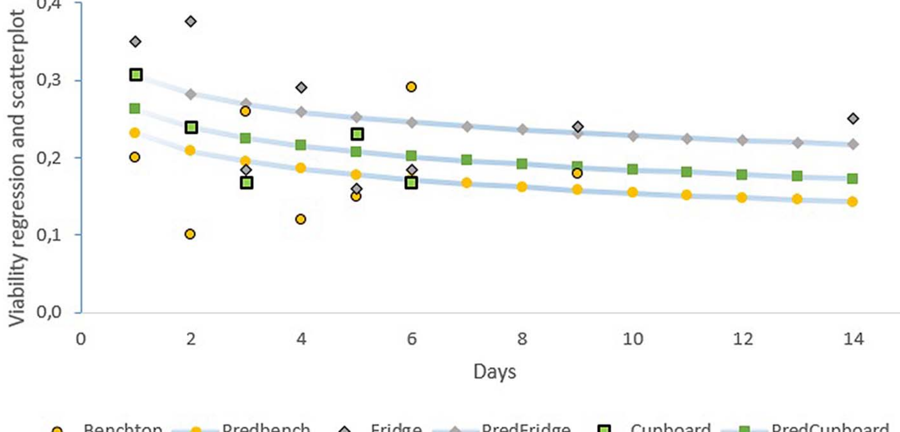

Fig. 3. Rate of decrease in the viability of *Cryptosporidium* oocysts on lettuce in different storage locations.

food container that had been inadvertently left unsealed. The lettuce sample wilted and shrivelled after a short time (< 1 day) of storage. Viability assessment indicated that all cysts were non-viable within only 20 h. This only occurred with a single sample on the benchtop; although providing interesting results, this investigation was not planned.

4. Discussion

The major finding from this study is that whereas both *Cryptosporidium* oocysts and *Giardia* cysts survive well when kept moist and refrigerated, survival of *Giardia* cysts was abrogated on lettuce at room temperature. Indeed, almost 50% die-off of *Giardia* cysts was recorded within the first 24 h.

The logistic regression showed that for *Giardia* cysts in water and on lettuce, time had a significant effect on viability, and for *Cryptosporidium* oocysts in water and on lettuce, time did not have a significant effect on viability during the limited period of time the viability was assessed.

For *Cryptosporidium* oocysts on lettuce, none of the storage locations differed significantly in terms of their effect on viability. For *Giardia* cysts, the cysts kept in the fridge had a significantly higher viability rate from the other storage locations.

Although various studies have investigated the survival of both these parasites in water and other environmental matrices, data on the survival and persistence of these transmission stages on foodstuffs are

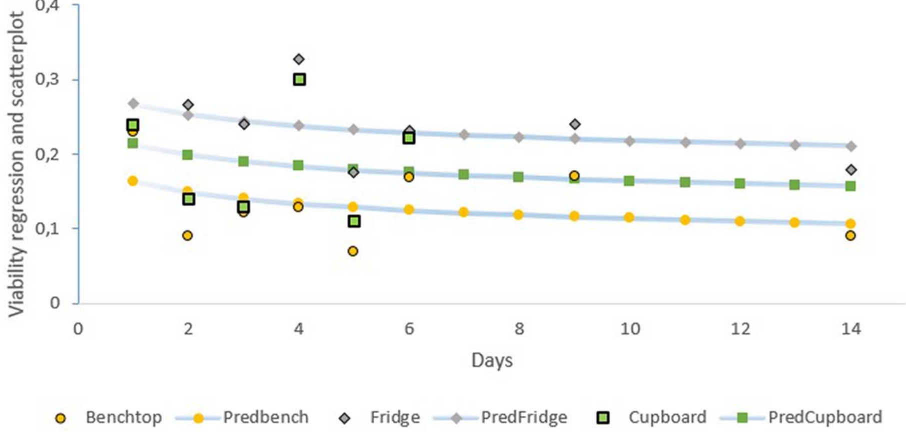

Fig. 4. Rate of decrease in the viability of *Cryptosporidium* oocysts on lettuce at different storage locations.

Table 4
Summary of publications on survival of *Giardia* cysts and *Cryptosporidium* oocysts in different environmental matrices.

Giardia viability assessments

Conditions	Viability assessment method	Viability after different exposure periods	Reference
Soil at 20 °C	Dye exclusion and bioassay	180 days/3% viability	Olson et al. (1999)
Soil at 4–5 °C	Dye exclusion and bioassay	180 days/almost no decline	Olson et al. (1999)
Soil during Norwegian winters (freeze–thaw cycles)	Dye exclusion	10–12 days/10% viability 75 days/ > 1% viability	Robertson and Gjerde (2006)
Lake water at 6–7 °C	Dye exclusion and bioassay	7 days/91% viability 28 days/1% viability	Cole et al. (1989)
Lake water at 17–20 °C	Dye exclusion and bioassay	7 days/12% viability	Cole et al. (1989)
Tap water at 20–28 °C	Dye exclusion and bioassay	7 days/2% viability 14 days/no cysts found viable	Cole et al. (1989)

Cryptosporidium viability assessments

Conditions	Viability assessment method	Viability	Reference
River water samples	Tissue culture assay	Infectivity decreased as temperature rises (from 4 to 23 °C)	Pokorny et al. (2002)
Cattle faeces	Bioassay	Survival time higher in cooler (above freezing) conditions	Li et al. (2010)
Distilled water at 15 °C	Bioassay and cell culture	Oocyst remained infective for 7 months.	Jenkins et al. (2002)
Environmental stresses (freezing, seawater, desiccation)	Dye exclusion	Freezing reduced viability greatly. Desiccation was detrimental.	Robertson et al. (1992)
On field crops at 20–30 °C	Not specified	Oocyst survival for < 3 days.	WHO – Wastewater use in agriculture (2006)

currently scarce and often unclear. As foodborne transmission has been frequently postulated to be important for both parasites (Smith et al., 2007), despite the discrepancy in recorded outbreaks, obtaining robust data on this is important and also identifying which factors may prolong survival and thus be more likely to result ingestion of viable transmission stages.

Table 4 lists some of the published survival experiments for both *Giardia* cysts and *Cryptosporidium* oocysts, and these illustrate that, in general, cooler temperatures, but not below freezing, promote survival of these transmission stages.

Similar results were obtained in the studies described here, and it was apparent that, in general, *Cryptosporidium* oocysts are more robust than *Giardia* cysts, particularly at room temperature.

Thus, to extrapolate our findings into a foodborne transmission scenario, especially with respect to fresh produce, it seems that temperature and humidity are critical factors regarding survival. Although fresh foods, such as salad, may act as a transmission vehicle for *Giardia* when contaminated by a food-handler shortly before consumption (as has been reported for at least two of the nine documented outbreaks of foodborne giardiasis), it seems that on-farm *Giardia* contamination or during the farm-to-fridge chain, in which the parasite is likely to be exposed to ambient temperatures for some periods, will be less likely to result in the potential for infection.

However, for *Cryptosporidium* oocysts, it appears that contamination of fresh produce, even very early in the farm-to-fork production chain, may result in viable parasites still being on the produce at consumption, even with a long-production chain; this is probably reflected in some of the outbreaks of foodborne cryptosporidiosis reported such as the outbreak in Finland involving 72 cases and associated with lettuce imported from the Netherlands (Pönka et al., 2009) and the UK-wide outbreak involving 300 cases (McKerr et al., 2012). For both these outbreaks, the widespread number of cases clearly indicate that foodhandlers at the serving or consumption places are not likely sources of the contamination.

Increased demand for fresh produce and current food trends advising consumers to increase their intake of vegetables and fruits, in combination with global sourcing and improved transport chains, may increase the possibility of fresh produce contaminated with parasite transmission stages being distributed more widely. In addition, and for *Cryptosporidium* in particular, it seems probable that oocysts that contaminate fresh produce, even at harvesting or before then, may remain in an infectious state on the foodstuff until it is consumed. Although those conditions that are optimal for transport of fresh produce (for example, for lettuce a temperature of 0 °C and a relative humidity of 98–100%; (Saltveit, 2014)) also tend to be ideal, or essential, for survival of *Giardia* cysts, most fresh produce is not transported under ideal conditions (Vigneault et al., 2009), although cold chain logistics are improving rapidly (Rodrigue and Notteboom, 2013). Whereas problems in the cold chain may be detrimental to the survival of *Giardia* cysts, *Cryptosporidium* oocysts are probably likely to be alive for as long as the fresh produce remains in a condition that makes it acceptable for sale.

In some countries transport, storage, and sale of fresh produce is less sophisticated. For example, in India, fresh produce is mostly sold either directly from the producer through open-air markets or street vendors, or, more usually, initially through mandi (trading hubs for agricultural produce), and, even at the mandi, the cold storage is usually insufficient (Ahmad and Siddiqui, 2015). In such conditions it would seem, again, that although *Cryptosporidium* oocysts contaminating the produce at the farm level may remain viable until consumption, this is less likely for *Giardia* cysts despite the farm-to-fork chain being shorter. This is particularly important for such regions where these parasitic infections are more prevalent, have a greater impact on the population, and where identification of the most important transmission routes is important for implementation of appropriate control measures.

In conclusion, although both *Giardia* cysts and *Cryptosporidium* oocysts survive well on fresh produce under cool, moist conditions, such are also ideal for transport and storage of fresh produce, if produce contamination occurs at the farm level (during production or harvesting) then *Cryptosporidium* oocysts are much more likely than *Giardia* cysts to survive until they reach the consumer. Thus, if we wish to ensure that our fresh produce does not become more likely to be contaminated with infectious parasitic transmission stages, it behoves us to implement improvements regarding removal or inactivation of parasite transmission stages, or, preferably, decreasing contamination at the farm level. Implementation of such measures should be con-

ducted in parallel with improvements in the fresh produce cold chain.

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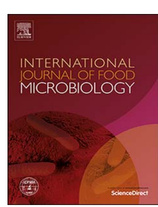

Checking the detail in retail: Occurrence of *Cryptosporidium* and *Giardia* on vegetables sold across different counters in Chandigarh, India

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ABSTRACT

Fresh produce has been recognized as a vehicle of infection for protozoan parasites, particularly *Cryptosporidium*, and, to a lesser extent, *Giardia*. For both parasites, outbreaks associated with fresh produce have been documented. Although documented outbreaks tend to be from industrialized countries, contamination of fresh produce with these parasites is a global issue. In developing countries, infections with these parasites are often endemic in the community, and basic infrastructure and hygiene measures may be inadequate, thus the likelihood of contamination of fresh produce with these parasites may be higher. Realization of the importance of this transmission route comes against a backdrop of raw salads and more Western culinary habits gaining a foothold, and fresh produce being encouraged as part of the diet due to their associated health benefits. However, if consumption of uncooked fresh produce is going to increase its market sector in India, it is important that it is safe. In this study, various types of fresh produce obtained from three types of vendors in Chandigarh, a major city in Northern India, were analyzed for contamination with *Cryptosporidium* oocysts and *Giardia* cysts using a method that has been previously validated in inter-laboratory spiking experiments. A total of 284 samples of different fresh produce items were analyzed, obtained from the different retailers situated in different societal layers of the city. The overall prevalence of contamination of fresh produce with these parasites was just under 11%, with 6% of the vegetables contaminated with *Cryptosporidium* oocysts, and 5% with *Giardia* cysts. Contaminated vegetables included turnip, cabbage, carrot, chili, coriander, cucumber, radishes, and tomatoes. Molecular analyses identified contamination with *Cryptosporidium parvum* and *Giardia duodenalis* of Assemblage A and Assemblage D, indicating that contamination from animals may be of relevance. Although the prevalence of contamination is similar to those reported in previous studies, the levels of contamination on some items of fresh produce were relatively high. Although the different socioeconomic areas of Chandigarh from which the samples were obtained was not associated with likelihood of contamination, fresh produce from supermarkets had heavier contamination with *Cryptosporidium* oocysts than fresh produce purchased through other sales outlets. The results are discussed in relation to the fresh produce chain and sales models in Chandigarh, both in terms of where contamination may occur and the potential importance of fresh produce as a transmission vehicle.

1. Introduction

Cryptosporidium spp. and *Giardia duodenalis* are among the most frequently occurring intestinal protozoan parasites in humans and animals worldwide (Fayer, 2004; Thompson and Monis, 2004; Thompson, 2004). Both parasites can cause diarrheal disease. Global studies have revealed that *G. duodenalis* and *Cryptosporidium* spp. are two of the most common etiological agents in pediatric diarrhea in developing countries, and are associated with mortality as well as morbidity (Kotloff et al., 2013; Platts-Mills et al., 2015).

The biology of *Cryptosporidium* and *Giardia* makes them suitable for transmission via fresh produce; they have a low infectious dose, a robust transmission stage, are small sized, and some genotypes have a zoonotic potential, giving the opportunity for contamination to occur

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from both animal and human sources (Robertson and Lim, 2011; Robertson and Fayer, 2012). Infected individuals also have a high excretion rate, ranging from > 5×10^3 to 9.2×10^5 oocysts per gram feces for *Cryptosporidium* (Goodgame et al., 1993) and 580,000 cysts per gram feces may be shed over a period of days or longer in the case of *Giardia* infection (Danciger and Lopez, 1975).

As fewer people are affected in a foodborne outbreak than in a waterborne outbreak, and may be more scattered geographically (see, for example, the UK outbreak described by McKerr et al. (2015)), a lack of prompt diagnosis may hamper epidemiological investigation. It is worth noting that the last major waterborne outbreak of giardiasis in Europe, in which over 1500 people were infected, took several weeks to be recognized as a waterborne outbreak (Robertson et al., 2006), and in the UK, the specific produce causing the outbreak was never detected.

Cryptosporidium and *Giardia* can contaminate food as a surface contaminant. Contamination with small numbers of infectious (oo)cysts in produce that receives minimal washing or treatment prior to ingestion, poses a threat to public health. Food products can be contaminated directly by cysts and oocysts in feces from humans and animals or via the environment, such as soil and water, and thus serve as vehicles for transmission, at any step in the farm-to-fork chain. For fresh produce, contamination may persist until infection as the production chain requires cool temperatures and moist conditions to maximize food freshness, and that also enhance survival of *Giardia* cysts; *Cryptosporidium* oocysts seem to be more tolerant to temperature changes on fresh produce (Utaaker et al., 2017). For foods such as, for example, bakery produce, storage conditions (dry, at room temperature) are likely to be deleterious to parasite survival. Although there has been discussion around *Cryptosporidium* oocysts surviving for longer in conditions of cool temperatures and high humidity, it has nevertheless been concluded that the likelihood of foodborne outbreaks occurring is no greater in cooler environments than anywhere else in the world (Robertson and Chalmers, 2013). Indeed, the foodborne transmission route is probably particularly relevant in places where infection is more likely underdiagnosed and underreported, and especially so in the developing countries where infrastructure and resources for investigation and reporting are limited. However, in such settings, where various intestinal infections are endemic, outbreaks caused by contamination of food or water may be more difficult to identify against a background of high infection.

Methods for detecting contamination of foodstuff by protozoans had been relatively poorly developed until recently, until the publication of ISO Method 18744 (ISO, 2016). However, this method is both expensive and time-consuming, and essential reagents must be stored refrigerated. To implement such methods for routine analysis in laboratories that are already poor in resources may be prohibitively expensive and impractical. Furthermore, considering the vast amount of fresh produce from different traders in the chain of retail events in a developing country's retail model, using lab analyses may provide scant information regarding tracking the sources of contamination. Nonetheless, these methods enable surveys to be conducted and an assessment of contamination levels to be made, and such data are essential for assessment of risk and determining the extent of significance of such contamination.

In this study, the aim was to analyze fresh produce sold at different retailers in Chandigarh, India for the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts, and to use molecular typing of parasites from positive samples as a further indication of the possible sources of contamination.

2. Materials and methods

2.1. Sampling site

Over a two-year period between February 2014 and February 2016, 284 vegetables were purchased at local mandi, street vendors, and supermarkets in Chandigarh, Northern India.

Chandigarh is a union territory of its two neighboring states, Haryana and Punjab, although not considered a part of either state. According to the State Agricultural Marketing Board of the Union Territory Chandigarh, Chandigarh has no major crop itself, and most fresh produce available in Chandigarh comes from these neighboring states. The Union Territory Chandigarh has only a limited area available for agriculture, and this land is being gradually diminishing with the expansion of Chandigarh city. In addition, farmers who keep a large number of dairy cattle utilize these areas to grow fodder for livestock (Chandigarh Administration, 2016a).

Chandigarh has only one principal Market Yard, and there are no official seasonal or other kinds of market yards, or any unregulated markets (C.S.A.B, 1961) Thus, most of the samples collected in this study came via the principal Market Yard, from where they are distributed to different trading hubs and retailers.

Chandigarh is a city undergoing rapid growth and development. The city is organized according to “phases”, which can be a proxy for socio-economic status, as reflected by density of inhabitants: Phase I (higher socio-economic status), Phase II (moderate socio-economic status), Phase III and non-sectorial villages (lower socio-economic status). In addition, Chandigarh is divided into sectors, based on the grid concept of the roads, and different sectors are also considered more or less affluent than others, according to the Chandigarh Master Plan – 2031 (Chandigarh Administration, 2016b).

2.2. Source of samples of fresh produce

The sampling strategy aimed at analyzing vegetables representing each sector of the city, including both rural and urban areas, the “phases” of the city, and to enable comparison of the three main sales types used for fresh produce in India: street vendors, mandi (see description below), and supermarkets. Nevertheless, due to access to vegetables and markets, the number of samples from each sector are non-uniform, as are the distribution of vegetable types and salespoints (see Table 1).

Among the total of 284 samples, 137 (48%) were obtained from individual street vendors, with either a stationary or mobile salespoint. The vendor handles the produce until it is purchased by the customer. In addition, 125 (44%) samples were obtained at mandi, which are local trading hubs for agricultural produce, and are arranged on a regular weekday basis in different sectors in Chandigarh. At the mandi, the local producer or salesperson brings his products and displays them for the customer. As with street vendors, only the vendor handles the foodstuffs until purchased by the customer.

The remaining 22 (8%) samples were obtained from modern supermarkets, where the vegetables are displayed for the customer to choose their preferred items, touching and handling the produce as they make their selection before paying at the counter.

The samples were collected from different areas (phases), or sectors, of Chandigarh, thus also representing vegetables from different socio-economic layers, as well as salespoints. Of the 284 samples, 119 were collected from Phase I sectors, 112 were collected from Phase II sectors, and 51 were collected from sectors in Phase III and non-sectorial villages.

The samples most probably all came from the same principal Market Yard, but were sold in different areas of the city, and thus under different conditions.

2.3. Fresh produce selected for analysis and their use in India

The vegetables to be analyzed were chosen according to the season of availability, with emphasis on those commonly consumed raw. These included coriander leaves, lettuce, radish, tomatoes, cucumber, fenugreek leaves, cabbage, chili, mint leaves, carrot, and turnip.

Table 1
Different types of vegetables and number of samples collected from different types of salespoints in Chandigarh.

Common name	Scientific name	No. of samples from mandi (whole sale markets)	No. of samples from street vendors	No. of samples from supermarkets	Total number of samples
Cabbage	*Brassica oleracea*	22	21	4	47
Carrot	*Daucus carota* subsp. *sativus*	8	14	3	25
Chili	*Capsicum annuum*	18	21	3	42
Coriander leaves	*Coriandrum sativum*	10	15	3	28
Cucumber	*Cucumis sativus*	26	22	4	52
Fenugreek leaves	*Trigonella foenum-graecum*	5	3	–	8
Lettuce	*Lactuca sativa*	1	1	–	2
Mint leaves	*Mentha spicata*	4	2	1	7
Radish	*Raphanus raphanistrum* subsp. *sativus*	7	7	–	14
Tomatoes	*Solanum lycopersicum*	22	30	4	56
Turnip	*Brassica rapa* var. *rapa*	2	1	–	3
Total		125	137	22	284

3. Sample analysis

3.1. Sample preparation

After collection, the vegetables were either refrigerated (for a maximum 48 h before analysis) or processed immediately (within 4 h). A portion of sample (for leafy vegetables, approximately 30 g leaves, and for vegetables with smooth surfaces 1–2 pieces weighing approx. 30 g) were put into stomacher bags (Seward BA6041/STR filter bag) and washed with 200 ml of glycine buffer for 4 min by hand manipulation. The eluate was transferred into 5 × 50 ml centrifuge tubes, and the bag was again washed with distilled water and the wash water used to fill up the tubes. The tubes were centrifuged for 10 min at 1550 rfg and, after aspiration of the supernatant, the pellets were concentrated into one tube per sample and refrigerated until the IMS step was performed, using the Dynabeads® GC-combo kit for isolation of *Cryptosporidium* oocysts and *Giardia* cysts, following the reduced cost protocol (Utaaker et al., 2015). The final suspension of 50 μl was pipetted onto a single-well slide (Novakemi ab) and air-dried at room temperature.

Dried samples were fixed with methanol and stained with FITC-conjugated monoclonal antibodies (mAbs) against *Cryptosporidium* oocyst walls and *Giardia* cyst walls (Aqua-glo™, Waterborne™ Inc., USA) and nuclei were stained with the fluorogenic DNA intercalator 4′,6 diamidino-2-phenylindole (DAPI) according to Smith et al. (2002). Samples were mounted with M101 No-Fade Mounting Medium, then each slide was covered by a glass coverslip and viewed promptly.

3.2. Microscopy

Microscopy was performed on a Leica DCMB microscope (× 20, × 40, and × 100 objectives), equipped with Nomarski differential interference contrast (DIC) optics. A blue filter block (480 nm – excitation, 520 nm – emission) was used for the detection of cysts and oocysts labelled with FITC-conjugated mAbs, and a UV filter block (350 nm excitation, 450 nm emission) was used for DAPI.

3.3. Enumeration

Each well was scanned systematically in an up-and-down or side-to-side manner, and *Cryptosporidium* oocysts and *Giardia* cysts were enumerated. When brilliant apple-green fluorescing ovoid or spherical objects within the appropriate size range for *Cryptosporidium* and *Giardia* were observed, magnification was increased to 40 ×, and the UV filter block was used for visualization of DAPI staining. Each (oo) cyst was recorded as DAPI-negative or DAPI-positive according to the presence of internal light blue staining.

Nomarski (DIC) objectives were used to examine morphological characteristics of the (oo)cysts.

A sample was considered positive if the (oo)cyst(s) exhibited typical fluorescence, with correct shape and size, and being DAPI-positive. If internal contents were lacking, but the morphometry was correct and the structure had a typical fluorescence, the (oo)cysts were described as "putative", as they lacked sufficient characteristics for definitive identification.

These "putative" samples were not considered for genotyping due to the lack of nuclei, but both putative and confirmed parasites were summed together for inclusion in the results as positive findings. In some of the samples containing numerous *Cryptosporidium* oocysts, the oocysts were occluded due to debris when examined under the UV-light, making DAPI-staining difficult to assess. However, due to the high numbers of parasites, these were also included for genotyping.

3.4. DNA extraction

Following microscopy, *Cryptosporidium* oocysts and *Giardia* cysts were retrieved from positive slides and DNA was prepared according to Robertson et al. (2009). Briefly, the coverslip from each slide was carefully removed and retained, whilst 25 μl aliquots of AL lysis buffer (Qiagen GmbH, Germany) were added to the slide wells, which were then scraped using a sterile scalpel blade. The buffer and scrapings were pipetted into a microcentrifuge tube. This process was repeated four times, and then the coverslip was replaced onto the slide that was then re-screened. For each slide, neither cysts nor oocysts could be detected after scraping.

The contents of each microcentrifuge tube containing slide scrapings were re-suspended in Tris-EDTA buffer and held at 100 °C for *Cryptosporidium* oocysts and 90 °C for *Giardia* cysts for 1 h, before the DNA was isolated using QIamp DNA mini kit (Qiagen GmbH), using an overnight step at 56 °C.

3.5. Molecular methods and sequencing

PCR was conducted with the primers and protocols listed in Table 2 for *Cryptosporidium* and *Giardia*. The products were separated and visualized by electrophoresis on 2% agarose gels using SYBRsafe® DNA gel stain under UV radiation. Positive samples were purified using.

High Pure PCR product purification kit (Roche Diagnostics), and sequenced on both strands at GATC Biotech, Germany. Sequences were examined using Geneious 10.1.2 software and sequence comparisons conducted using NCBI BLAST. New sequences have been submitted to GenBank and have been allocated accession numbers KY967229, KY967230, KY967231, KY967232, KY967233.

Table 2
Positive results from microscopy (per sample) and PCR results.

Produce	Place of sampling	Microscopy results per 30 g sample; *Giardia* cysts	Microscopy results per 30 g sample; *Cryptosporidium* oocysts	PCR results *Giardia* SSU[a]	PCR results *Cryptosporidium* *SSU*[b] *COWP*[c]
Cabbage	Vendor	1 putative cyst			
Cabbage	Mandi		4 putative oocysts		
Cabbage	Supermarket		> 1000 oocysts		*C. parvum*[b] KY967229
Cabbage	Mandi		350 oocysts		NA
Carrot	Vendor	1 cyst		NA	
Chili	Mandi		46 oocysts		NA
Chili	Mandi		> 100 oocysts		NA
Chili	Mandi	4 cysts		NA	
Chili	Vendor	8 cysts		NA	
Chili	Mandi	4 putative cysts			
Chili	Mandi	16 cysts		NA	
Coriander	Vendor	2 putative cysts			
Coriander	Vendor	2 putative cysts			
Coriander	Mandi	8 putative cysts			
Coriander	Vendor		2 putative oocysts		
Coriander	Mandi		1 putative oocyst		
Cucumber	Vendor	1 cyst		Assemblage D[a] KY967232	
Cucumber	Mandi		70 oocysts		*C. parvum*[b,c] KY967230 and KY967231
Cucumber	Supermarket		> 1000 oocysts		NA
Cucumber	Supermarket		4 oocysts		NA
Radish	Mandi		1 putative oocyst		
Turnip	Mandi	1 putative cyst			
Tomatoes	Vendor	1 putative cyst			
Tomatoes	Vendor	3 cysts		Assemblage A[a] KY967233	
Tomatoes	Mandi		1 putative oocyst		
Tomatoes	Mandi		1 putative oocyst		
Tomatoes	Mandi		1 putative oocyst		
Tomatoes	Vendor		> 100 oocysts		NA
Tomatoes	Vendor		1 oocyst		NA
Tomatoes	Supermarket		246 oocysts		NA
Total positive samples		13 samples (1–16 cysts)	17 positive samples (1– > 1000 oocysts)	*Giardia* Assemblages identified in two samples	*Cryptosporidium* species identified in two samples

NA – no amplification.
[a] Hopkins et al. (1997).
[b] Xiao et al. (1999).
[c] Yu et al. (2009).

3.6. Statistics

A database of results was created in excel and parametric and non-parametric (ANOVA and Mann-Whitney *U* tests) were used to compare mean and median values. Contingency table analysis was used to test for associations between positive results and other factors. Statistical significance was considered for *p* values < 0.05.

4. Results

4.1. Occurrence of Cryptosporidium and Giardia on fresh produce

Of the 284 vegetable samples analyzed, 30 (ca. 11%) were found to be contaminated with either *Cryptosporidium* oocysts or *Giardia* cysts; 17 (ca. 6%) samples were contaminated with just *Cryptosporidium*, 13 (ca 5%) samples were contaminated with just *Giardia*, and none of the samples were found to be contaminated with both parasites (Table 2).

Over 10% of samples of coriander (5/28, ca. 18%), chilis (6/42, ca. 14%), and tomatoes (8/56, ca. 14%) were contaminated. For cabbages and cucumbers, the contamination rate was approximately 9% (4/47) and approximately 8% (4/52) respectively; see Fig. 1.

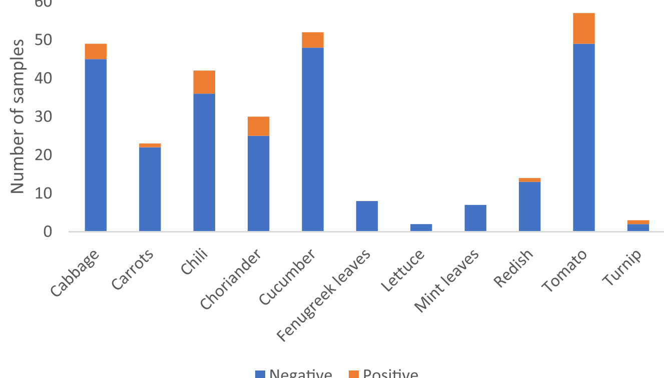

Fig. 1. Proportion of vegetable samples that are positive and negative for *Giardia* and *Cryptosporidium* according to type of produce.

4.2. Concentrations of parasites per sample

The extent of contamination with both *Giardia* and *Cryptosporidium* on positive produce was generally low (Table 2), with 10 out of 13 *Giardia*-positive samples having < 5 cysts detected and 9 out of 17 *Cryptosporidium*-positive samples having < 5 oocysts detected. The highest number of *Giardia* cysts detected was 16 (per 30 g sample of chili), and six samples of produce (two tomato, two cabbage, 1 cucumber and 1 chili) were considered to be very heavily contaminated with *Cryptosporidium*, having over 100 oocysts per sample. The median

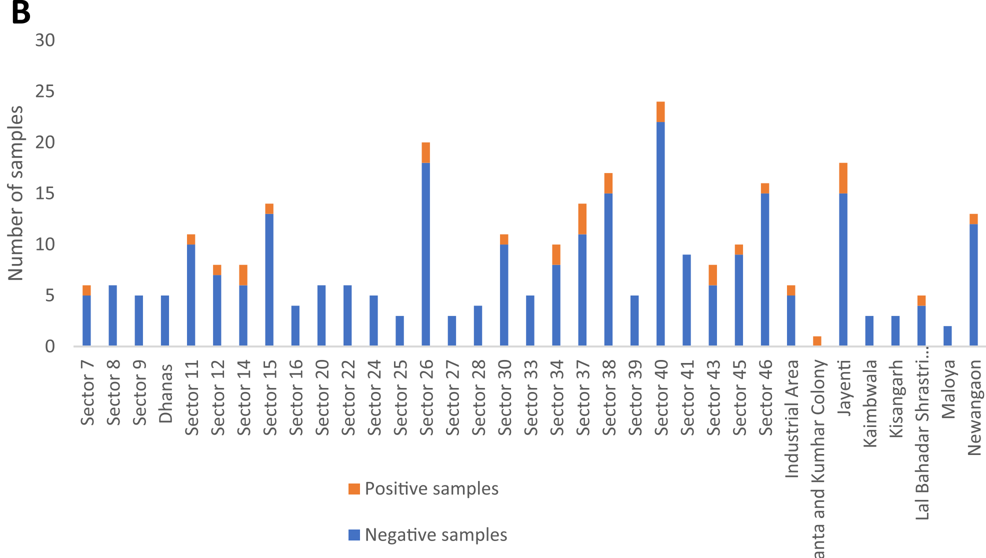

Fig. 2. *Giardia* and *Cryptosporidium* on vegetables sold in Chandigarh: (A) areas where vegetables were sold; (B) proportion of vegetables contaminated according to location.

numbers of *Giardia* cysts on produce was 2, for *Cryptosporidium* oocysts the median was 4. No significant difference was found when comparing median numbers of (oo)cysts using the Mann-Whitney *U* test.

4.3. Distribution of positive samples by area of Chandigarh

The occurrence of *Cryptosporidium* and *Giardia* on vegetables sampled from different phases of Chandigarh were compared using a two-rows by three columns Freeman-Halton test, and the difference in occurrence between the areas was not significant (Fig. 3; $p = 0.58$).

The distribution of positive samples according to sector where the samples were obtained is shown in Fig. 2A and B. The areas of lower socio-economic status did not have greater occurrence of contamination than other sectors.

It is worth mentioning that of those samples with high levels of contamination, the sample with the highest *Giardia* contamination was obtained from a sector of low socio-economic status, and among the 6 samples with over 100 *Cryptosporidium* oocysts per sample, 5 were from a sector of moderate socio-economic status and 1 was from a sector of low socio-economic status.

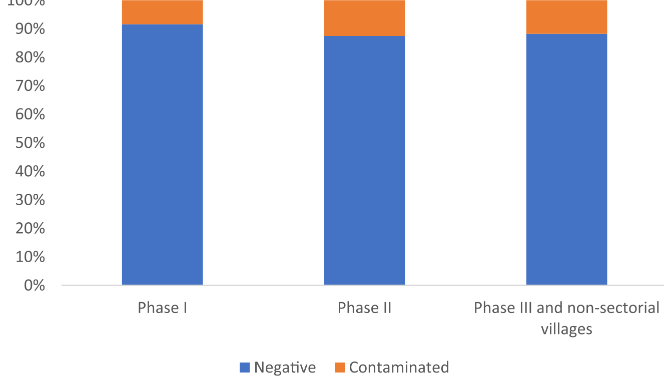

Fig. 3. Distribution of samples according to area and the proportion of contaminated and negative samples. The values are normalized.

4.4. Distribution of positive samples by vendor type

Of the 22 samples from the supermarket, 4 (18%) were

contaminated. Of the 125 samples from the mandi, 15 (ca. 12%) were contaminated, and of the 137 samples from the street vendors, 11 (ca. 8%) were contaminated. Thus, supermarket bought produce was no less likely to be contaminated than produce from mandi or street vendors.

With respect to the more highly contaminated samples (higher numbers of oocysts or cysts), the sample with the highest number of *Giardia* cysts was obtained from a mandi, whereas of the produce more highly contaminated with *Cryptosporidium* oocysts, three were obtained from a supermarket (with two of these having over 1000 oocysts per sample), two were from a mandi, and one was from a street vendor.

The median number of *Cryptosporidium* oocysts per positive sample from supermarkets was 623, from mandi was 2.5, and from street vendors was 2. The median values from *Giardia* cyst and *Cryptosporidium* oocyst contamination from vendors and mandi were compared by Mann-Whitney *U* test, and the difference was not significant.

None of the samples from the supermarkets were contaminated with *Giardia* cysts. Thus, the extent of contamination with *Cryptosporidium* oocysts of positive produce from supermarkets was significantly higher than *Cryptosporidium*-positive produce from traditional retailers as determined by Mann-Whitney U test.

4.5. Molecular analyses from positive samples

4.5.1. Genotyping of Giardia

The DNA isolated from 6 *Giardia* positive samples was run through PCR reactions using several primer sets targeting different genes, including triosephosphate isomerase (TPI), glutamate dehydrogenase (GDH) and small-subunit RNA (SSU), however DNA amplification was largely unsuccessful. Results from which usable sequences could be obtained derived only from 2 samples and only at the SSU gene (Table 2). Comparison using BLAST indicated that the closest hits (both 96%) were Assemblage A (closest hit GenBank Accession number LN811460.1) and D (JQ245138.1).

4.5.2. Species identification of Cryptosporidium

The DNA isolated from 10 *Cryptosporidium*-positive samples produced amplification and usable sequences from 2 samples using both the SSU and COWP primers; both samples were found to be *C. parvum*.

5. Discussion

One of the most important findings from this study is that a relatively high proportion of fresh produce on the market in Chandigarh (just under 11%) were contaminated by protozoan parasites, with 6% contaminated with *Cryptosporidium* oocysts and 5% with *Giardia* cysts.

Given that Chandigarh is generally regarded as one of the cleanest cities in India (Chandani, 2016), this seems a high proportion. However, studies from some other low- or middle-income countries have indicated similar or higher levels of contamination (e.g., Ebrahimzadeh et al. (2013), Maikai et al. (2013), Said (2012)). In more developed countries, the proportion of fresh produce contaminated with these parasites tends to be lower (e.g., Rzeżutka et al. (2010), Robertson and Gjerde (2001), Dixon et al. (2013)).

An important difference, however, between the results obtained from our study in Chandigarh and the data from other studies is that some of our samples had very high levels of contamination. Studies from developing countries often do not often report the extent of contamination, but when quantitative data are provided, the maximum contamination per sample is generally a few (oo)cysts (e.g., Fallah et al., 2012; Al-Shawa and Mwafy, 2007). Whereas the majority of our samples had low levels of contamination, several samples had considerably higher levels of contamination for *Cryptosporidium*.

Many studies from developing countries investigating contamination of fresh produce with protozoan parasites have not used established methods and do not provide recovery efficiency data. This makes interpretation of the results difficult, and it is likely that both the extent of contamination and contamination levels may be under-estimated. Our study utilized an established protocol that has been validated in different laboratories (Utaaker et al., 2015); the recovery efficiency is around 30–50%.

The stage along the farm-to-fork continuum at which the fresh produce in our study became contaminated is impossible to determine from our data. However, this information is important for identifying where preventive measures should be implemented. Various publications have shown that irrigation water may be a source of contamination of crops (Chaidez et al., 2005; Thurston-Enriquez et al., 2002) (Amorós et al., 2010), although *Cryptosporidium* and *Giardia* were not found in water samples from an irrigation canal in Thailand (Chuah et al., 2016). Irrigation in the Punjab and Haryana states is largely from ground water being pumped directly onto growing crops from tube wells (Pandey, 2016). Ground water is usually relatively protected from contamination and may be considered less likely to be the source of *Cryptosporidium* oocysts and *Giardia* cysts. However, canal irrigation (e.g. the Upper Bari Doab canal in Punjab) is also an important irrigation source in this region. With canal irrigation, floodwater is carried to the agricultural areas, and high levels of contamination may occur, particularly if there is the potential for the canals to be contaminated with sewage. Based on our results, investigation of irrigation water in these areas is recommended.

Water, as a potential source of contamination, can come into contact with fresh produce not only during cultivation, but also during washing. It is common practice in Asia for street vendors and mandi salespeople to keep a bucket of water alongside their wares and to moisten their displays so that they appear fresh and appealing. A study from Vietnam examined water from 200 such buckets and found *Giardia* cysts in 17 buckets (median concentration of 20 cysts per ml), and *Cryptosporidium* oocysts in 9 buckets, with a median value of 10 oocysts per ml (Tram and Dalsgaard, 2014). Although such buckets of water were not analyzed in this study, they were noted as potential sources of contamination.

People in the chain from field to fork who handle the produce might also be sources of contamination. These include people working in the fields harvesting the crops, people packing the crops for transport to the city, and the various links in the sales chain within the city, as well as the last link in the chain, the salesperson themselves, or other customers handling the produce. Our results from supermarkets suggest that in situations where various customers may handle the goods, but not necessarily purchase them, contamination may occur. Indeed, the more people who handle the produce the greater the potential risk of contamination. Whereas salespeople have a vested interest in not selling their customers contaminated goods, this does not apply to other customers who handle goods.

Molecular analyses providing information on species and genotype may provide further clues regarding sources of contamination, particularly whether the parasites are likely to be from humans or animals. The information from our molecular analyses does not rule out either human or animal sources of contamination with *Cryptosporidium*. A study of *Cryptosporidium* species in human infections in Chandigarh indicated that *C. hominis* was the most prevalent species (75%) followed by *C. parvum*, with a prevalence of 25% (Sharma et al., 2013). Information on *Cryptosporidium* species in animals in around Chandigarh is scant, but our own studies have not indicated widespread *C. paruvm* infections among animals, although subgenotypes of *C. parvum* detected in livestock in this area (unpublished data) are the same as those reported in human infections by Sharma et al. (2013). The source of contamination of fresh produce could thus be from either dirty hands or infected livestock or other animals.

Similarly, for *Giardia*, either humans or animals could be the source of contamination, although the finding of Assemblage D as a contaminant of cucumber indicates contamination from canine feces is most likely, and probably does not pose a risk to human health. The

sample containing these cysts was obtained from a vendor selling produce from an open pushcart in the street, giving ample opportunity for contamination from dog feces (Mahajan, 2014). Assemblage A, which was found contaminating tomatoes, is infectious to both humans and a range of other animals, and thus it is not possible to narrow down the likely source of contamination.

The widespread occurrence of these parasites on vegetables in Chandigarh along with the high levels of contamination and some samples being shown to be contaminated with species or genotypes known to be infectious to humans, indicate that there is the potential for infection transmission by consumption of contaminated fresh produce, although whether the contaminating parasites were infective at point of purchase was not investigated, and the question remains regarding the threat from this contamination to public health.

An expert elicitation study (Hald et al., 2016), estimated that the proportion of cases of cryptosporidiosis and giardiasis caused by the foodborne route in Southeast Asia was 0.10 and 0.13, whereas equivalent data from Western Europe were 0.10 for *Cryptosporidium* and 0.11 for *Giardia*. Unlike with other foodborne pathogens, this study indicated that the importance of the foodborne route of infection was quite similar across regions, but, in general, was rather low, with water and human-to-human contact being of greater importance (Hald et al., 2016). Interestingly, based on this expert elicitation, foodborne transmission seemed to be considered slightly more important for *Giardia* than *Cryptosporidium* consistently across regions. However, this may not reflect that there are fewer foodborne cases of cryptosporidiosis than giardiasis, but that infections from animals are possibly of greater importance with *Cryptosporidium* than *Giardia*. Although foodborne outbreaks of these infections have been reported much more frequently from wealthier countries than poorer countries, this does not mean such foodborne outbreaks do not occur in less wealthy countries. However, with high levels of endemicity, it may be difficult to determine when an outbreak associated with a specific vehicle of infection is occurring, and following up such an outbreak requires enormous resources and prioritization of effort, both of which are unlikely to be available in developing countries.

The results of our study thus support the potential for foodborne transmission, but should not be read to indicate that this transmission mode is more important than any other. However, given the levels of contamination that we found, it seems probable to us that foodborne transmission may be more likely to occur in this setting than in European countries or other wealthy environments.

India is a hierarchical society, both within and between families, and also other social groups. Particular social groups tend to cluster together in terms of where they live in a city, and our intention was to investigate whether particular strata of the society were more or less likely to be exposed to these parasites through fresh produce than others. In Chandigarh, such investigations are relatively easy, as the city was originally planned for a differential pattern of density.

Due to cheaper housing, Phase III areas and non-sectorial villagers are characterized by high population pressure. They also have unsanitary conditions, flood problems, poor garbage disposal, disposal of livestock dung into open drains, and discharge of untreated sewage (Chandigarh Administration, 2016b).

Although it was expected that fresh produce from Phase III areas and non-sectorial villages would have higher rates of contamination than from other areas, this was not the case. This could be because the city as a whole has a relatively high population density, with approximately 9300 persons per km^2, according to the census organization of India (Indian 15th National Census Survey, 2012). This means that the demarcations between the phases of the city are not very clear, as the sectors spread into each other. Furthermore, as most of the fresh produce supply passes through a single principal Market Yard in Chandigarh (Chandigarh Administration, 2016c), and from there is distributed to all the different sectors, the origin of the fresh produce is alike, regardless of hierarchical position at point of sale.

One difference that our study did bring out, however, was that contamination of fresh produce with *Cryptosporidium* was at higher levels (significantly greater numbers of oocysts) in supermarkets than in street vendors or mandi. Interestingly, modern food retailing has apparently not been highly successful in India, with most Indian shoppers, regardless of disposable income bracket, preferring to buy fresh produce from a street trader or mandi than a supermarket (Economist, 2014). Price and convenience are often cited reasons, but it has also been suggested that the benefits of supermarket shopping is in choice range rather than quality, and our data would seem to support this. Why produce in supermarkets should have higher levels of *Cryptosporidium* oocysts, than fresh produce at other salespoints is not clear, but may reflect greater handling potential via more people (customers).

India is the second largest producer of fruits and vegetables in the world, and this production is considered a labor-intensive and high-risk activity (Sachdeva et al., 2013). The fresh produce business in India has numerous infrastructure problems, including insufficient cold storage, unreliable transport, poor compliance with safety standards, insufficient quality control, lack of research and development, challenges in labeling etc., according to the Ministry of Food Processing Industries, G.o.I (2006), New Delhi. This situation hampers both prevention and traceability of contamination, and indicates that the best measure to avoid infection by consumption of contaminated produce is to maintain good hygienic practices in the preparation of fresh produce by proper rinsing and washing.

Although our study produced useful data acquired by standard and recognized methods, there were challenges. Inhibitors such as polysaccharides, polyphenols, pectin, xylan, and chlorophyll from the plant material in our samples may have hampered the PCR reactions (Wei et al., 2008). Furthermore, due to practical and logistical issues between the collaborating laboratories, the period between elution and examination of slides was often prolonged. Parasites may have degenerated during storage and the formation of other microorganisms during the storage and transport period may have had a deteriorating effect on the DNA in terms of both degeneration and formation of inhibitors.

In conclusion, our study found that contamination with *Cryptosporidium* oocysts and *Giardia* cysts of fresh produce on the market in Chandigarh was relatively frequent. In some cases, high numbers of oocysts and cysts were detected. Molecular studies suggest that some of this contamination probably originates from animals, but species and genotypes infectious to humans were also indicated.

Of particular note is that supermarkets, which are generally considered more modern, and thus (intuitively) safer, were no less likely to sell contaminated produce and produce was highly contaminated. This may reflect the greater handling in supermarkets, where the customers are able to touch and handle the produce themselves.

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IV

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Is drinking water making waves in Chandigarh? Occurrence of Cryptosporidium and Giardia in potable water sources.

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Abstract:	Contamination with Cryptosporidium and Giardia of potable water in a city in Northern India was assessed. A protocol modified from the standard ISO protocol was tested and showed to produce comparable recovery efficiencies at a substantial cost reduction. This protocol was used for analysing 71 ten-litre samples of potable water from different areas of Chandigarh, where sampling locations were divided into groups according to socio-economic status and population density, which also partially equates with level of infrastructure. Samples were collected during (n=29) and outside the monsoon season (n=42). Of all samples analysed, 16 (22.5%) were positive for Cryptosporidium and/or Giardia. Numbers of parasites per sample was generally low, although one sample contained large numbers of Giardia cysts. Molecular analyses tended to be unsuccessful, although Giardia cysts of Assemblage B and C were identified. No association with season was detected, but an association with location of water supply was identified. Samples from areas with lowest infrastructure were not associated with higher levels of contamination, but samples from the middle level were significantly more likely to be contaminated. Results indicate that even in a modern city like Chandigarh, contamination of potable water with protozoan parasites remains a significant risk.

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Is drinking water making waves in Chandigarh? Occurrence of *Cryptosporidium* and *Giardia* in potable water sources.

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Abstract

Contamination with *Cryptosporidium* and *Giardia* of potable water in a city in Northern India was assessed. A protocol modified from the standard ISO protocol was tested and showed to produce comparable recovery efficiencies at a substantial cost reduction. This protocol was used for analysing 71 ten-litre samples of potable water from different areas of Chandigarh, where sampling locations were divided into groups according to socio-economic status and population density, which also partially equates with level of infrastructure. Samples were collected during (n=29) and outside the monsoon season (n=42). Of all samples analysed, 16 (22.5%) were positive for *Cryptosporidium* and/or *Giardia*. Numbers of parasites per sample was generally low, although one sample contained large numbers of *Giardia* cysts. Molecular analyses tended to be unsuccessful, although *Giardia* cysts of Assemblage B and C were identified. No association with season was detected, but an association with location of water supply was identified. Samples from areas with lowest infrastructure were not associated with higher levels of contamination, but samples from the middle level were significantly more likely to be contaminated. Results indicate that even in a modern city like Chandigarh, contamination of potable water with protozoan parasites remains a significant risk.

Keywords: *Cryptosporidium* oocysts, detection, Drinking water, *Giardia* cysts, public health.

Introduction

The World Health Organization (WHO) estimates that about 1.1 billion people globally drink unsafe water (Kindhauser and WHO 2003), and the vast majority of diarrhoeal diseases in the world (88 %) is attributable to unsafe water, poor sanitation, and general lack of hygiene. Waterborne parasitic protozoan diseases result in 842.000 deaths per year and 1.7 billion cases of diarrhoea (WHO 2002) (Checkley et al. 2015), and the morbidity and mortality caused by waterborne diarrhoeal disease make them one of the planet`s biggest environmental health threats to many populations (Gadgil 1998).

Cryptosporidium and *Giardia* are two of the most common aetiological agents of childhood diarrhoea in developing countries, causing morbidity as well as mortality (Kotloff et al. 2013, Platts-Mills et al. 2015), and an expert elicitation found that waterborne transmission accounts for, proportionally, 0.37 *Cryptosporidium* infections and 0.35 *Giardia* infections in South-East Asia, making the waterborne route one of the most important exposure pathways (Hald et al. 2016). Between 2011 and 2016, out of 381 waterborne outbreaks of cryptosporidiosis and giardiasis that have been documented, all of them are reported from developed parts of the world, where there have been significant advances in setting up surveillance systems and reporting (Baldursson and Karanis 2011). The most frequent aetiological agent was *Cryptosporidium* spp., reported in 63% of the outbreaks, while *Giardia* was implicated in 37%, making it clear that these parasites contribute substantially to waterborne diarrhoeal disease.

A questionnaire study from Bolivia reported that only 30% of the respondents associated dirty water with diarrhoea, and that diarrhoea was perceived as a normal childhood occurrence (Quick et al. 1997). This perception would indeed substantially contribute to underreporting of outbreaks and disease in developing countries, where these parasites are endemic, the disease burden caused by these pathogens is more common, and waterborne infections are seldom reported. This is not

because they do not occur, but because there is virtually no organized system for identification, description, and analysis of these events (Efstratiou et al. 2017), and the proportion that are specifically waterborne is seldom identified. Indeed, those regions that are probably most affected by *Cryptosporidium* and *Giardia* do not have the resources to identify or provide any reports of waterborne outbreak events, and nor do they have the resources available to implement routines regarding surveillance and monitoring that can be the basis of risk assessment and introducing interventions where needed.

The lack of data around these fundamental questions was the basis for this study in which we investigated the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in potable water samples in different seasons in and around Chandigarh, a city in Northern India.

Materials and Methods

Validation and use of a reduced-cost protocol for analysis

Methods of analysis of water for contamination with *Cryptosporidium* and/or *Giardia* cysts have been developed, validated, and adopted throughout many countries in the developed world and the ISO 15553 standard is probably the most commonly used approach in Europe (ISO 2006). This is a costly method, and one of the most expensive steps in the protocol incorporates the use of immunomagnetic separation (IMS). The IMS procedure is also an essential step in other standard methods of water analysis for these parasites, such as US EPA Method 1623.

As we have previously been successful modifying another ISO Method, 18774 (ISO 2016) that also incorporates IMS in order to reduce the cost (Utaaker et al. 2015), a similar approach was used here to enable us to have the resources to analyse more samples.

In order to validate the modified method for use with potable water, ten 10-L samples of tap water

were spiked with EasySeed™ (BTF Pty Ltd, Australia) that contains 100 *Cryptosporidium* oocysts and 100 *Giardia* cysts, sorted in suspension. Five samples were analysed according to the ISO 15553:2006 protocol (for which our laboratory is accredited by the National Accreditation authority), and the other five samples were analysed by the modified method, in which, rather than using the IMS reagents as in the ISO Method, the IMS was performed according to the method published by Utaaker et al, (2015). In brief, following concentration of the sample into an 5-7 ml volume following filtration and centrifugation, only 20 µl volume of each type of beads was used, rather than 100 µl, and the buffers were modified such that rather than using 1 ml of each of the buffers provided with the IMS kit (Dynabeads®: *Cryptosporidium/Giardia* Combo Kit, Idexx Laboratories), a smaller volume was used augmented with buffers as described in Utaaker et al, (2015).

Field Sampling

From August 2014 to February 2016, 71 potable water samples of 10 L each were collected in and around Chandigarh, Northern India.

Chandigarh is divided into sectors, distributed into three phases that are based on population density; 39 samples were collected from phase I sectors, which has the lowest population density, 12 samples were collected from phase II, which has a higher density than phase I, and 20 samples were collected from phase III and non-sectorial villages surrounding the city.

The samples were collected into 10 L plastic containers directly from a public drinking water source, that was either supplied by a tubewell or from a water tank transported into the area. After collection, the containers were taken directly to the laboratory and processed immediately.

Analysis of water samples

The samples were analysed for the presence of *Giardia* cysts and *Cryptosporidium* oocysts using the modified protocol of the ISO 15553 (2006) standard, which had been tested prior to use in the same

laboratory and provided recovery rates comparable to the ISO method (see sections 2.1 and 3.1). The reagents were used as according to Utaaker et al, (2015).

The water samples were first filtered within 2 hours after collection at the parasitology laboratory at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh through Millipore Isopore membrane filters with a pore size of 2 µm using a Watson-Marlow 520 Bp Profibus pump. Following filtration, the filters were placed in 50 mL centrifuge tubes that were then filled with sample water and stored at 4°C. These sample tubes containing filters were transported from PGIMER to the parasitology lab in Norway for the final stages of the analysis (IMS, immunofluorescent antibody testing (IFAT), and any molecular analyses).

In Norway, the filters were washed as according to 15553 (ISO, 2006), and the eluate was poured into 50 mL centrifuge tubes. The tubes were centrifuged at 1550 rfg for 10 minutes and, following aspiration of the supernatant, the remaining pellets were concentrated into one tube before the IMS step was performed, using the Dynabeads® kit for isolation of *Cryptosporidium* oocysts and *Giardia* cysts, but using the reagents as described in Section 2.1 (Utaaker et al, 2015). Following dissociation of the beads by vortexing under acidic conditions, the final suspension of 50 µL was pipetted onto a single-well slide (Novakemi ab, Sweden) and air-dried at room temperature.

Dried samples were fixed with methanol and stained with FITC-conjugated monoclonal antibodies (mAbs) against *Cryptosporidium* oocyst walls and *Giardia* cyst walls (Aqua-glo™, Waterborne™ Inc, USA) and nuclei were stained with the fluorogenic DNA intercalator 4´, 6 diamidino-2-phenylindole (DAPI) according to Smith et al., (Smith et al. 2002). Samples were mounted with M101 No-Fade Mounting Medium, then each slide was covered by a glass coverslip and viewed promptly by fluorescent microscopy.

123 **Microscopy and Enumeration**

124 Microscopy was performed on a Leica DCMB microscope (x 20, x 40 and x100 objectives), equipped
125 with Nomarski differential interference contrast (DIC) optics. A blue filter block (480 nm – excitation,
126 520 nm – emission) was used for the detection of cysts and oocysts labelled with FITC-conjugated
127 mAbs, and a UV filter block (350-nm excitation, 450-nm emission) was used for investigating DAPI-
128 staining.

129 Each well was scanned systematically in an up-and-down or side-to-side manner at 20X, and
130 *Cryptosporidium* oocysts and *Giardia* cysts were enumerated. When brilliant apple-green fluorescing
131 ovoid or spherical objects within the appropriate size range for *Cryptosporidium* and *Giardia* were
132 observed, magnification was increased to 40X, and the UV filter block was used for visualization of
133 DAPI staining. Each (oo)cyst was recorded as DAPI-negative or DAPI-positive according to the
134 presence of internal light blue staining.

135 Nomarski (DIC) objectives were used to examine morphological characteristics of the (oo)cysts.

136 A sample was considered positive if the (oo)cyst(s) exhibited typical fluorescence, with correct shape
137 and size, and being DAPI-positive. If internal contents were lacking, but the morphometry was
138 correct and the structure had a typical fluorescence, the (oo)cysts were described as "putative", as
139 they lacked sufficient characteristics for definitive identification.

140 Both putative and confirmed parasites were summed together for inclusion in the results as positive
141 findings. In some of the samples the (oo)cysts were also quite occluded due to debris when examined
142 under the UV-light, making DAPI- staining difficult to assess.

143 **Molecular analyses**

144 **Extraction of DNA from oocysts**

145 Following microscopy, *Cryptosporidium* oocysts and *Giardia* cysts were retrieved from positive slides
146 and DNA was prepared according to Robertson et al., (2009). Briefly, the coverslip from each slide

was carefully removed and retained, whilst 25 µl aliquots of AL lysis buffer (Qiagen GmbH, Germany) were added to the slide wells, which were then scraped using a sterile scalpel blade. The buffer and scrapings were pipetted into a microcentrifuge tube. This process was repeated four times, and then the coverslip was replaced onto the slide that was then re-screened. For each slide no cyst or oocysts could be detected after the procedure.

The contents of each microcentrifuge tube containing slide scrapings were re-suspended in Tris-EDTA buffer and held at 100°C for *Cryptosporidium* oocysts and 90°C for *Giardia* cysts for one hour, before the DNA was isolated using QIamp DNA mini kit (Qiagen GmbH), using an overnight step at 56°C.

PCR

The samples were run through several PCR reactions using different primer sets listed in supplementary table 1, and two sequences from PCR targeting the SSU gene of *Giardia* were obtained. The PCR products were purified using ExoSAP-IT® (Affymetrix USB) and sent to GATC Biotech for sequencing. The sequences were analysed using Geneious 10.2.1©, compared by NCBI Blast and submitted to GenBank.

Statistics

The recovery efficiencies of the spiked *Cryptosporidium* and *Giardia* by the modified method were compared with those obtained by the ISO 15556 method by linear regression.

The results of sample analysis were collated in an excel database. A chi-square test was used to compare contamination according to season of collection of the samples and a Freeman-Halton test was used to compare contamination according to the location of the site of sample collection.

Results

Recovery efficiency using modified method

The recovery of *Giardia* cysts was significantly higher using the reduced cost approach (reduced cost approach; mean = 77.4%, ISO 15556 approach; mean recovery efficiency = 61.6%; p = 0.017).

Cryptosporidium recovery efficiency was not significantly different when the methods were compared (p = 0.320).

These results (Figure 1) were the basis for using the reduced cost approach for analysing the samples.

Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in potable water samples from different locations in Chandigarh.

Of the 71 samples analysed, 16 (22.5%) were positive for either *Cryptosporidium* oocysts or *Giardia* cysts. Seven samples (9.9%) were contaminated with *Cryptosporidium* oocysts, and ten samples (14.1%) were contaminated with *Giardia* cysts. One sample contained both parasites.

An overview of the results with location of sampling site, season of sample collection, number of parasites per 10 L sample, and the results from molecular analyses are summarised in table 1.

Comparison of results according to season indicated that there was no association of positive results with the monsoon season or any other season (p= 0.995); Figure 2

However, location of sample collection area had a significant effect on the probability of a sample being contaminated (PA and PB = > 0.001), with samples more likely to be positive if obtained from locations in Phase II of Chandigarh (Figures 3 and 4). Although Phase III samples were no more likely to be positive than samples from Phase I, in a slum area in Phase III one highly positive sample was found containing large numbers of *Giardia* cysts. Genotyping of *Giardia* cysts from this location indicated that Assemblage B *Giardia* cysts were present. The two sequences from *Giardia*-positive samples have been issued Accession numbers MF 150151 and MF 150152.

Amplification of the target genes from *Cryptosporidium*-positive slides was unsuccessful in all cases.

191 **Table 1:** Positive results from potable drinking water sources analysed for contamination with

192 *Cryptosporidium* oocysts and *Giardia* cysts.

Phase	Season	Source	Microscopy results per 10 L sample; *Cryptosporidium* oocysts	Microscopy results per 10 L sample; *Giardia* cysts
Phase I (lowest population density and highest level of infrastructure)				
	Monsoon	Sector 9	1 oocyst	
	Winter/Spring	Sector 17	3 oocysts	8 cysts
	Winter/Spring	Sector 14	1 oocyst	
	Winter/Spring	Sector 27		1 cyst
	Monsoon	Sector 30	10 oocysts	
	Monsoon	Sector 8		1 putative cyst
Phase II				
	Summer	Sector 38	1 putative oocyst	
	Summer	Sector 38		1 putative cyst
	Monsoon	Sector 38		1 cyst
	Monsoon	Industrial area		2 cysts
	Monsoon	Sector 34		2 cysts
	Winter/Spring	Sector 38		136 cysts Assemblage C MF 150151[a]
	Winter/Spring	Sector 40	1 oocyst	
Phase III and non-sectorial villages (highest population density and lowest level of infrastructure)				

	Monsoon	Kishangarh,	1 putative oocyst	
	Winter/Spring			>1000 cysts
		Janta & Kumhar Colony		
	Winter/Spring	Janta & Kumhar Colony		3 cysts Assemblage B MF 150152[a]
Total		**16**	**7**	**10**

a: GenBank Accession number

Discussion

The main result of this study is that the potable water samples analysed from in and around Chandigarh were rather likely to be contaminated with *Cryptosporidium* oocysts and/or *Giardia* cysts. Although other studies with a similar design have found varying occurrences, in these reports the positive samples are often from raw and untreated water sources; e.g. in Norway a survey reported a prevalence of positive samples 16.5% for *Cryptosporidium* and 11.5% of *Giardia* (Robertson and Gjerde 2001), but the samples were from untreated surface water. Similarly, a study from Ankara, Turkey, investigated municipal water supply, wells, river water and untreated dams. No contamination was found in the municipal water supply, but the wells, dam and river samples gave an overall occurrence of 5.8% of samples being *Giardia* positive (Bakir et al. 2003). In countries that are perhaps more comparable with India, a study encompassing analysis of water samples from Malaysia, Thailand, Philippines, and Vietnam reported only a single potable water sample (from the Philippines) containing both *Cryptosporidium* and *Giardia*, whereas neither parasite was detected in potable water samples from the other three countries (Kumar et al. 2016). Nevertheless, earlier studies from North America have reported both *Cryptosporidium* and *Giardia* contamination in

potable drinking water; a survey from US revealed a 17% prevalence of *Cryptosporidium*, and no *Giardia* (Rose et al. 1991), and a survey from Canada reported a prevalence of 18.2% *Giardia* cysts and 3.5% *Cryptosporidium* oocysts (Wallis et al. 1996). Although our findings from Chandigarh's drinking water may give some cause for concern, particularly in relation to results from potable water in other Asian countries, they are not abnormally high compared with some earlier reports from some other places in the world. It should be noted that Chandigarh is a modern city, and has been ranked as one of the most advanced and cleanest cities in India (Chandani 2016). Thus, our findings from here should not be taken as representative for the whole country.

One obstacle to surveying water supplies for these parasites in developing countries is the expense of the technique. In this study, the ISO-standard and a modified alternative were compared prior to sample collection, and the modified version was found to perform with compatible recovery rates at a lower cost and was thus used in the survey. This method could be further tested and validated in multi-laboratory ring trials, and could offer a cheaper alternative for laboratories or research projects with constrained budgets.

Climate is considered to be likely to impact the occurrence of protozoan parasites as contaminants in drinking water, particularly with respect to extreme weather events. A meta-analysis indicated that the likelihood of contamination of fresh surface water with *Cryptosporidium* oocysts and *Giardia* cysts was significantly increased during extreme weather events (Young et al. 2015); thus, it may be expected that during the monsoon season contamination would be increased. In Chandigarh, there are three distinct seasons: from April to mid-July is summer with hot, dry weather, leading to occasional heat waves, and a mean average rainfall of about 30mm/month; the water demand for domestic supply and irrigation during these months is very high. Monsoon season, from mid-July to mid-September, is when Chandigarh receives moderate to very heavy rainfall, with an average of 222 mm/month, with a peak in precipitation in July and August, with 288 mm and 304mm, respectively. The winter, when a small part of the rainfall also occurs, averages on 33 mm/month. Spring season is

235 from mid-September to March, with mild temperatures and less precipitation (O.P. Singh 2012)
236 (Indian meteorological department). Chandigarh receives an annual average rainfall of approximately
237 1000 mm, most of which is confined to the monsoon season (JNNURM 2006).

238 In this study, the seasons had no apparent effect on contamination, and samples taken in the
239 monsoon season, in particular, were no more likely to be positive than samples taken during other
240 seasons. However, this result may reflect the unstable precipitation during the period of sampling as
241 during this period, the seasons and precipitation differed to that which is normal for Chandigarh.
242 During the monsoon season in 2014, the Chandigarh district only received about 45% of its normal
243 rainfall, and 2014 was ranked as "dry to extremely dry" (IMD, 2015). Furthermore, 2015 was the third
244 warmest year in India recorded since 1901, and although the year was considered within the normal
245 range in terms of overall precipitation, the rain came in unexpected seasons. During the summer,
246 Chandigarh received three times more than the normal rainfall, but during the usual monsoon
247 season, there was a 30% deficiency in expected precipitation, and the winter season was below
248 normal in terms of rainfall (IMD, 2016).

249 Nevertheless, from our data no specific season appeared to be more associated with contamination
250 than others, and the risk of contamination is known to be associated with many variables. It is
251 possible that season and precipitation are not major drivers for contamination in this area or that
252 insufficient samples were analysed to reveal a seasonal pattern, particularly in the light of the
253 unusual and unexpected weather patterns during sampling.

254 Although season was not associated with contamination of drinking water, the location where the
255 water was collected from did affect the likelihood of the sample being contaminated. Chandigarh city
256 is an area planned for a specific number of inhabitants, and, additionally, was originally planned for a
257 differential pattern of density. Like most cities in India, Chandigarh is overpopulated, with a current
258 population density of approximately 9300 persons per km^2 (census organization of India). Citizens of
259 Chandigarh are unevenly distributed throughout the city, in so-called phases numbered from I to III,

260 with increasing densities from phase I, situated in the northern part of the city, to phase III, which is
261 at the south end. The city is also divided into sectors, in a grid-pattern based on the roads, and
262 beyond those, there are so-called non-sectorial villages and slum colonies. These villages and slum
263 colonies lack much of the infrastructure and sanitary facilities of the planned sectors. Due to cheaper
264 housing, the Phase III areas and non-sectorial villagers are characterized by high population pressure,
265 with consequences such as unsanitary conditions, flood problems, poor garbage disposal, disposal of
266 livestock dung into open drains, and discharge of untreated sewage (Chandigarh Master Plan –
267 2031).

268 Water table elevation studies have revealed that the flow of ground water is from the north to the
269 south within the city (O.P. Singh 2012), which, in turn, means that the groundwater flows from phase
270 I through phase II and III. Thus, not only does Phase III have the highest population density, but also
271 the groundwater has run through the other two sectors before reaching this stratum. This
272 stratification within the city also results in the social layers being more exposed and accessible for
273 investigation, as particular social groups tend to cluster together in terms of where they live in a city,
274 and this is probably particularly demarcated in India, which is a hierarchical society. Although our
275 results indicate significant differences in likelihood of contamination of the potable water according
276 to Phase, our expectation that Phase III areas and non-sectorial villagers would have higher rates of
277 contamination of drinking water compared with other areas was not the case. Despite the factors of
278 poor infrastructure and lack of basic hygiene, the Phase III areas and villages did not have a
279 significantly higher contamination rate than those in Phase I. Nevertheless, it should be noted that
280 one sample with a very high level of contamination was from a slum colony.

281 The similarity in contamination between Phase I and Phase III may reflect the fact that the city as a
282 whole has a relatively high population density, such that the demarcations between the phases of
283 the city are not very clear, and the water sources are distributed on a relatively small area. However,

this does not explain why sampling sites in Phase II were significantly more likely to show contamination.

Previously, the water supply of Chandigarh was based on tubewell sources alone. As the population has increased, so has the demand for water supply, and the underground source alone is currently not sufficient to meet the city's requirements. This gap is met by tapping surface water from the Bhakra Main Line. The city has also been divided into 7 zones for the purpose of distribution, and it is interesting that the local water works supply water to distinct zones of density in the city as well. The three first zones supply to sectors 1-30, which have lower population densities. Zones four and five supply water to phase II, a more densely populated area than the previous 30, and the last two zones supply water to Manimajra, an area situated just outside Phase I. The non-sectorial villages and Phase III are not supplied with water from waterworks, according to the City Development Plan, and thus these non-sectorial villages do not have access to piped drinking water supply, sewer systems, or storm water drainage (Administration 2016). Thus, drinking water supplies may be driven in to non-sectorial villages and Phase III, and thus the residents here may actually be provided with a cleaner water supply than those people living in the, arguably wealthier, Phase II areas.

However, as the highest contamination was found in a water tank in a Phase III slum area, it is likely that individual tanks have the potential to be hot-spots of contamination and sources of infection. They are more prone to on-the-spot contamination, and as they are stagnant, enclosed sources of drinking water, and removal of contamination due to flow-through may be difficult. However, in this study, only 8 tanks were sampled, of which 2 samples were positive and thus further studies are warranted.

It was hoped that molecular methods would supply further information on the source of contamination, and also on the likelihood of infection to people. Although most of the PCR studies did not result in DNA amplification, the two successfully genotyped samples were of *G. duodenalis*, Assemblage B and Assemblage C. Whereas Assemblage B is usually associated with human infection,

Assemblage C is commonly associated with canids and is not associated with human infection. This Assemblage C *Giardia* was identified in a contaminated public water source in a Sikh temple. The public frequently visits these temples, and it is common to wash and drink from the taps. This means the source could have been someone handling an infected dog, or could have come directly from one of the many dogs in Chandigarh, the population of which is currently increasing (Victor 2013).

Conclusions

The major factors that lower the significance and impact of diarrhoeal diseases on public health are good sanitation, plentiful availability of good quality water, adequate disposal of human and animal excrement, and public education in hygienic practices (Gadgil, 1998). The high morbidity caused by *Giardia* and *Cryptosporidium* in developing countries has been attributed to factors including the multiple exposure routes and cycling through domestic animals, high environmental burden, and resistance of the (oo)cysts against common water disinfectants (Slifko et al. 2000).

The rapid population growth in Chandigarh, which places further stress on an already scarce water supply, has led to solutions that may not be sustainable or appropriate on a long-term basis. The highest proportion of contaminated water was found in more densely populated areas, and the sample containing the highest number of parasites was taken from a water tank in a slum area. It seems that the safe potable water with respect to contamination with protozoans is currently largely restricted to the higher socioeconomic layers in Chandigarh.

It is noteworthy that in situations with poor quality drinking water, the benefits derived from improving only the sanitation tend to be larger than from improving only the quality of drinking water (Esrey 1996). However, combined efforts to improve both knowledge and standards of drinking water by establishing surveillance systems as a first step, and implementing adequate reporting systems and appropriate epidemiological surveillance, would be expected to give positive results in combating parasitic protozoa and improving the health of the population.

Although the results of our study do not implicate the potable water supply as a source of intestinal protozoan infections for the citizens of Chandigarh, or provide evidence that it is a major transmission vehicle, they do indicate that it can be a potential source of infection, particularly in less wealthy areas of the city. Further research that identifies how and where contamination occurs would be of value, such that appropriate barriers can be implemented.

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Competing interests

The authors state that they have no competing interests.

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Figure legends

Fig 1: *Cryptosporidium* and *Giardia* recovery from spiked water samples according to method.

Fig 2: Occurrence of *Cryptosporidium* and *Giardia* in drinking water sources in Chandigarh according to season. The values are normalised.

Fig 3: Occurrence of *Cryptosporidium* and *Giardia* in water according to phases in Chandigarh. The values are normalised.

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Figure

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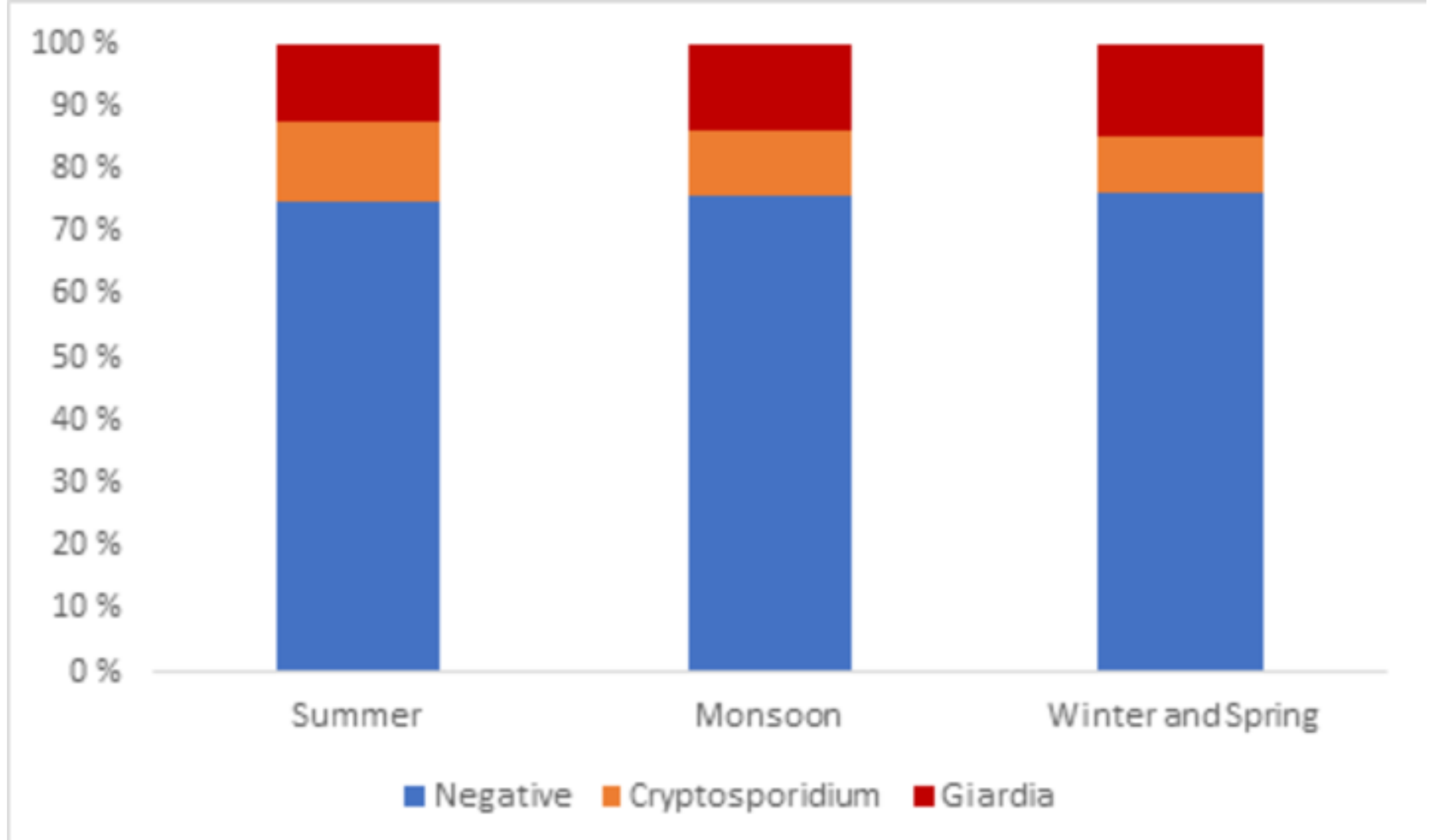

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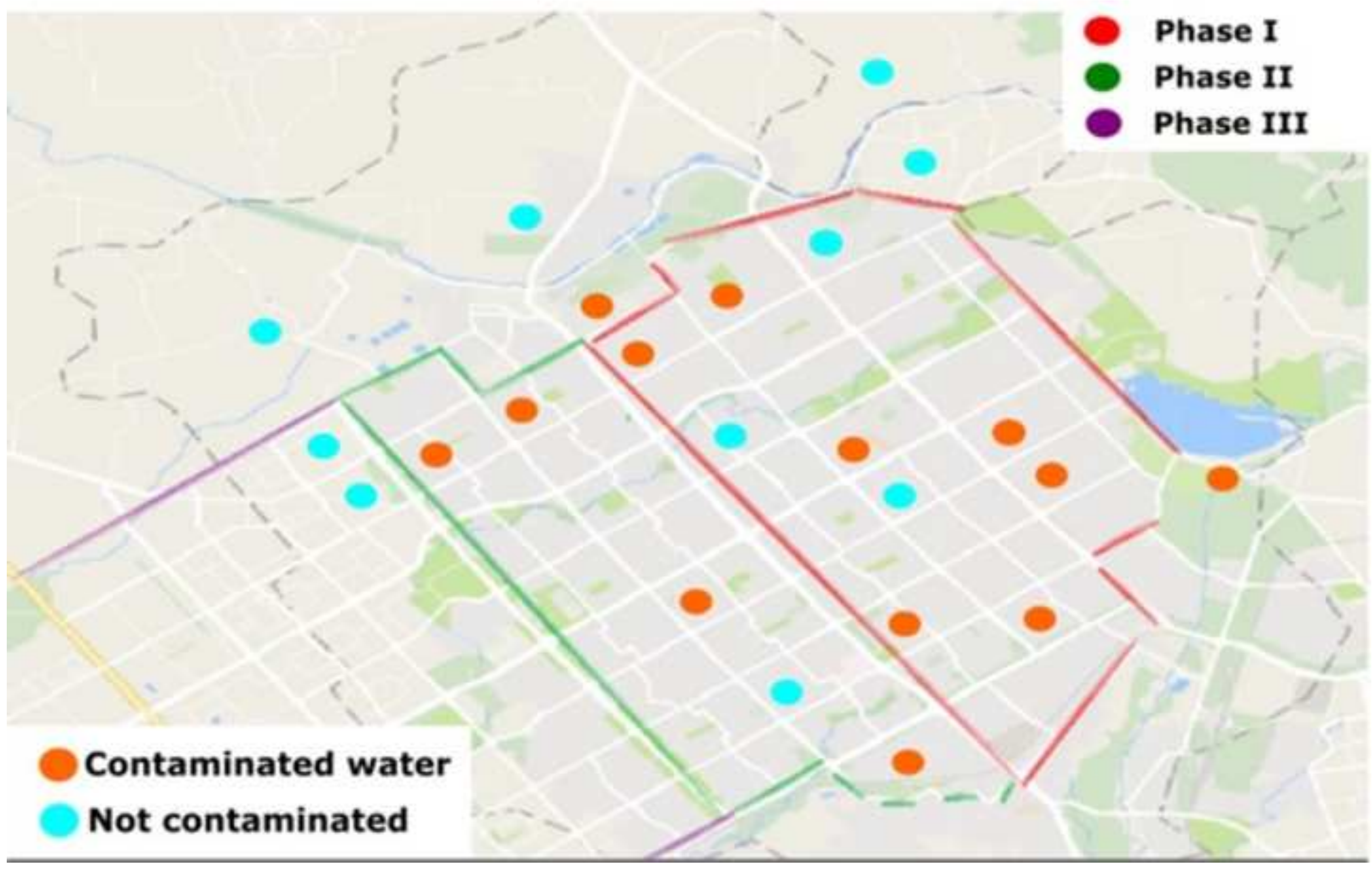

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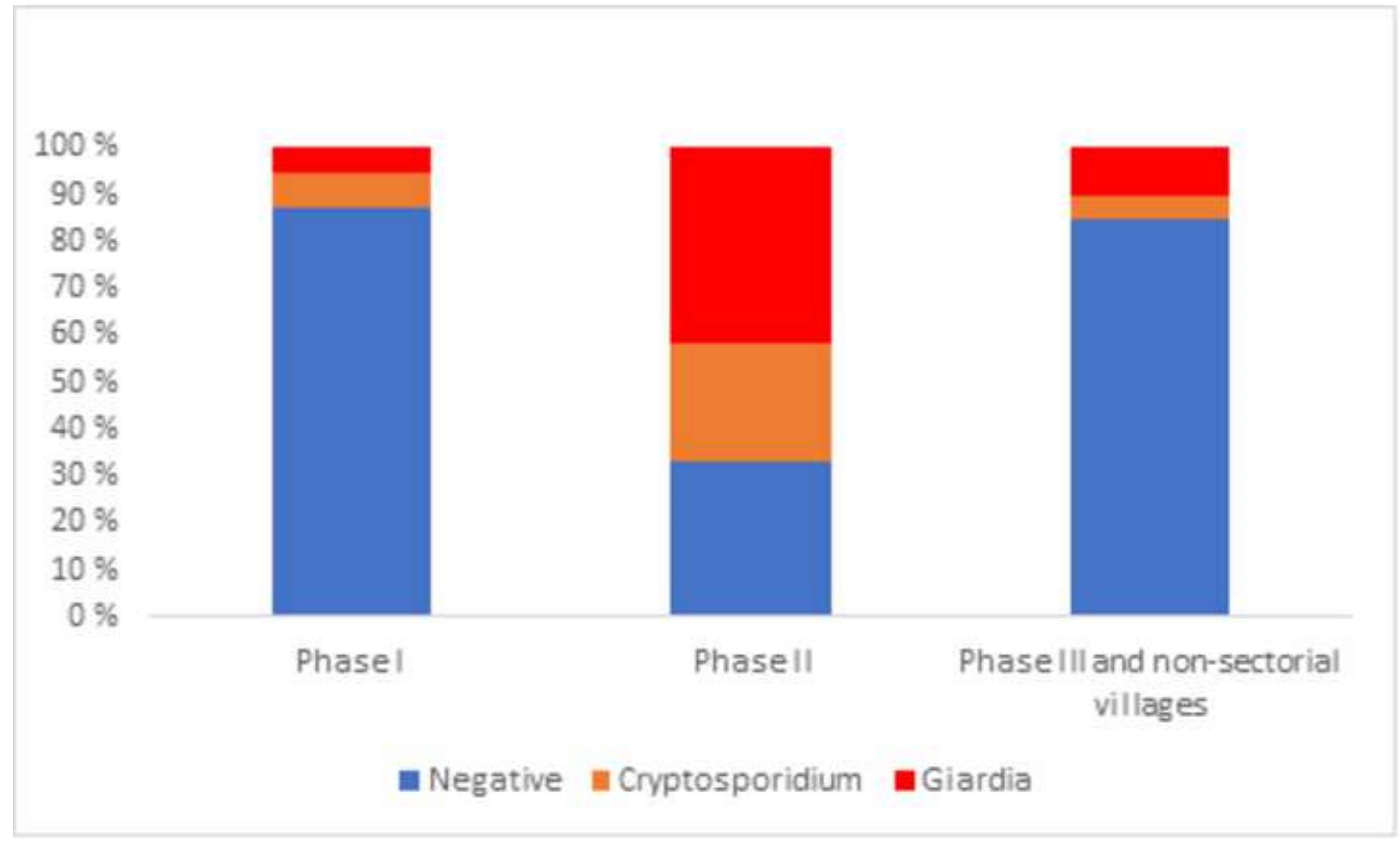

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Supplementary table 1. PCR conditions for detection of *Giardia* and *Cryptosporidium.*

Locus	Amplicon length	Primer	Cycle conditions		References
Giardia					
Small Subunit (SSU) rRNA	292	1st amplification	96 °C, 5 min		Hopkins et al., 1997
		F: 5′-CATCCGGTCGATCCTGC-3′	96 °C, 30 sec		Read et al., 2002
		R: 5′-AGTCGAACCCTGATTCTCCGCCAGG-3′	59 °C, 40 sec	40 x	
			72 °C, 40 sec		
			72 °C, 7 min		
	175	2nd amplification	96 °C, 5 min		
		F: 5′-GACGCTCTCCCCAAGGAC-3′	96 °C, 30 sec		
		R: 5′-CTGCGTCACGCTGCTCG-3′	55 °C, 40 sec	50 x	
			72 °C, 30 sec		
			72 °C, 7 min		
Triosephosphate Isomerase (TPI)	605	1st amplification	95 °C, 10 min		Sulaiman et al., 2003
		F: 5′-AAATYATGCCTGCTCGTCG-3′	94 °C, 45 sec		
		R: 5′-CAAACCTTYTCCGCAAACC-3′	50 °C, 45 sec	45 x	
			72 °C, 60 sec		
			72 °C, 10 min		
	563	2nd amplification	95 °C, 10 min		
		F: 5′-CCCTTCATCGGNGGTAACTT-3′	94 °C, 45 sec		
		R: 5′-GTGGCCACCACVCCCGTGCC-3′	50 °C, 45 sec	45 x	

			72 °C, 60 sec		
			72 °C, 10 min		
Glutamate Dehydrogenase (GDH)		1st amplification:	94 °C, 15 min		Read et al., 2004
		F: 5′-TCAACGTYAAYCGYGGYTTCCGT-3′	94 °C, 45 sec	50 x	Robertson et al., 2006
		R: 5′-GTTRTCCTTGCACATCTCC-3′	54 °C, 45 sec		
			72 °C, 45 sec		
			72 °C, 10 min		
Beta Giardin (BG)	753	1st amplification:	95 °C, 15 min		Caccio et al., 2002
		F: 5′-AAGCCCGACGACCTCACCCGCAGTGC-3′	94 °C, 30 sec	35 x	Lalle et al., 2005
		R: 5′-GAGGCCGCCCTGGATCTTCGAGAC GAC-3′	60 °C, 30 sec		
			72 °C, 60 sec		
			72 °C, 10 min		
	511	2nd amplification	95 °C, 15 min		
		F: 5′-GAACGAGATCGAGGTCCG-3′	95 °C, 30 sec	40 x	
		R: 5′-CTCGACGAGCTTCGTGTT-3′	53 °C, 30 sec		
			72 °C, 60 sec		
			72 °C, 10 min		
Cryptosporidium					
SSU rRNA	860	1st amplification	95 °C, 15 min		Xiao et al, 1999
		5′-GGAAGGGTTGTATTTATTAGATAAAG-3′	94 °C, 45 sec	50 x	
		5′-AAGGAGTAAGGAACAACCTCC A-3′	55 °C, 45 sec		
			72 °C, 60 sec		
			72 °C, 10 min		

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Goats in the city: prevalence of Giardia and Cryptosporidium in extensively reared goats in Northern India

--Manuscript Draft--

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Abstract:	Abstract Background Various characteristics of goats mean they are highly suitable livestock for backyard rearing of people with limited resources. They are a popular livestock choice in India where they are often kept to supplement an already scarce income. In these settings, hygiene and sanitation standards tend to be low, and weakens the interface between human and animals, thus making the barrier for zoonotic and anthroponotic infections more likely to occur. Results This article describes an investigation of the occurrence of Cryptosporidium spp. and Giardia duodenalis in goats being reared in different settings in urban and peri-urban areas in Northern India, and addressed the zoonotic potential of these important protozoan parasites shed from goats living close to humans. The overall prevalence of Giardia was 34.3% and Cryptosporidium was 0.5%; the relatively low prevalence of Cryptosporidium infection may reflect that most samples were not derived from young animals. The prevalence of Giardia excretion was found to be similar to that reported in other studies. However, although other studies have reported a predominance of non-zoonotic Assemblage E in goats, in this study potentially zoonotic Assemblages predominated (Assemblage A (36 %) and Assemblage B (32 %)). Conclusions This indicates that in situations and areas where goats and humans are living in close proximity, there may be sharing of intestinal parasites. This can be detrimental for both host species.
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Goats in the city: prevalence of *Giardia* and *Cryptosporidium* in extensively reared goats in northern India

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Keywords: Backyard livestock, *Cryptosporidium*, developing countries, *Giardia*, goat, One Health, zoonosis.

Abstract

Background

Various characteristics of goats mean they are highly suitable livestock for backyard rearing of people with limited resources. They are a popular livestock choice in India where they are often kept to supplement an already scarce income. In these settings, hygiene and sanitation standards tend to be low, and weakens the interface between human and animals, thus making the barrier for zoonotic and anthroponotic infections more likely to occur.

Results

This article describes an investigation of the occurrence of *Cryptosporidium* spp. and *Giardia duodenalis* in goats being reared in different settings in urban and peri-urban areas in northern India,

and addressed the zoonotic potential of these important protozoan parasites shed from goats living close to humans. The overall prevalence of *Giardia* was 34.3% and *Cryptosporidium* was 0.5%; the relatively low prevalence of *Cryptosporidium* infection may reflect that most samples were not derived from young animals. The prevalence of *Giardia* excretion was found to be similar to that reported in other studies. However, although other studies have reported a predominance of non-zoonotic Assemblage E in goats, in this study potentially zoonotic Assemblages predominated (Assemblage A (36 %) and Assemblage B (32 %)).

Conclusions

This indicates that in situations and areas where goats and humans are living in close proximity, there may be sharing of intestinal parasites. This can be detrimental for both host species.

Background

Cryptosporidium spp and *Giardia duodenalis* are among the most common enteric parasites of domestic animals, humans and wildlife [1], and some species and assemblages are recognized to have considerable zoonotic potential. They are also two of the most common aetiological agents of paediatric diarrhoea in developing countries, where poverty and density are high, and hygiene standards is low, and these protozoans are associated with mortality as well as morbidity [2, 3]. Given the high prevalence of giardiasis and cryptosporidiosis in people living in underdeveloped communities, these diseases were included in the WHO "neglected disease initiative" in 2004 [4].

Several epidemiological studies from India have shown that human giardiasis is prevalent throughout the country, and community-based studies from northern India revealed prevalence rates from 5.5% to 70%, with highest rates in low socioeconomic groups in Chandigarh [5]. Giardiasis has a significant public health impact due to the high prevalence and disease burden of infection, and its association with causing major outbreaks. The effects of *Giardia* infection on growth and cognitive functions of infected children, particularly in developing countries, where people are exposed to other insults to their health, is of particular importance [6]. *Giardia* is also a common infection in livestock and companion animals, and sometimes, but not always, associated with disease [7-9]. The zoonotic potential of *Giardia* is controversial, with some Assemblages apparently host-specific, while others are more promiscuous in host proclivity [10]. In northern India, a study found the prevailing Assemblages among humans to be Assemblage B, which is associated with anthroponotic transmission, followed by assemblage A, which has been found in a wide range of mammals [11].

Cryptosporidiosis is a diarrhoeal disease found in humans and animals worldwide [12], and can be caused by several species and genotypes of *Cryptosporidium* [13]. In humans, *C. hominis* and *C. parvum* are the aetiological agents responsible for most infections [14], whereas *C. hominis* only infects humans and *C. parvum* primarily infects humans and ruminants. Strong links between contact with infected livestock and humans have been demonstrated in *Cryptosporidium* infections [15, 16].

In India, *Cryptosporidium* have been reported with prevalence ranging from 3.8% in patients in northern India, with the majority of infections attributed to *C. hominis* [17], to 39.7% in the rural population [18].

In areas with inadequate infrastructure of water supply and sanitation, and where animals are roaming free and often in close contact with humans, the importance of *Giardia* and *Cryptosporidium* as One Health parasites remains to be fully defined.

The potential for transmission of zoonotic agents between humans and animals is amplified when the interface between them is close, and the management of animals and hygienic setting is poor. A huge part of the populations of developing countries live under such conditions, and often backyard livestock are relied upon to provide extra income or food. Goats are highly suitable livestock for backyard rearing of people with limited resources as their grazing preferences enable them to feed on weeds, shrubs and other plants that other domestic animals refuse, their small size means they require less space than larger animals and they are easier to work with than large ruminants and are cheaper to buy and maintain [19]. Also, unlike sheep, goats have a high capacity for adapting to extreme climatic conditions, and are therefore are particularly valuable in arid and semi-arid regions to which sheep are unable to adapt so readily. Although on a global scale, sheep are much more common than goats, in India the goat population is more than double the sheep population, being 133 million goats and 63 million sheep in 2014 [20].

There are very few studies available that report on prevalence of infections with the potential to be transmitted between goats and their owners or consumers in areas where they are most likely to exert the greatest impact on each other. Indeed, in a review from 2009 on *Cryptosporidium* and *Giardia* in sheep and goats [21], the vast majority of data were reported from sheep. We identified one report of *Cryptosporidium* and *Giardia* among goats in India [22] with 35 % prevalence of *Cryptosporidium* and 20 % prevalence of *Giardia* found among goats in Odisha in Eastern India.

However, molecular analyses were not conducted and therefore the zoonotic potential was not ascertained.

The study described in this article aimed at determining the prevalence and zoonotic potential of *Cryptosporidium* and *Giardia* in small-scale goat farms and backyard livestock goats in urban and peri-urban areas in northern India.

Material and methods

Sampling

A total of 207 faecal samples from goats in Chandigarh, Punjab and Haryana were collected with consent from the owners. The samples were collected from both urban “village” areas in Chandigarh, and peri-urban villages in the neighbouring states of Punjab and Haryana. These goats were mainly kept for meat production.

Singha Devi, Jayenti and Kurali are small towns and villages located in the S.A.S Nagar district of the state Punjab. The district has a population of close to a million, with an almost even dispersion in rural and urban areas. The population density is 909/ Km^2 [23] The goat population of the S.A.S Nagar district has been estimated to be 6330 [24].

Saketri is a village in the Panchkula district of the Haryana state. The district has a population of about half a million, with a density of 625/ Km^2, and the population is almost evenly distributed in rural and urban areas [23]. The goat population of Panchkula has been estimated to be 8199 [25].

The city Chandigarh has a population of about one million, with almost all of its inhabitants living in urban areas. The population density of the city is 9258/Km^2. Kansal and Maloya are so-called non-sectorial villages associated with the city, but with poorer infrastructure and lower socioeconomic levels compared with the city itself [26]. Sector 38 West is the location of the slum colony Rajiv. Slum settlements have grown in the past decades in Chandigarh, especially in the periphery of the city,

where poor families tend to settle due to cheap housing. It was in these areas goats were kept as well. The goat population of Chandigarh has been estimated to be 805. (Department of Animal husbandry and Fisheries, Chandigarh Administration, [27]

The samples (each approximately 5-10 g) were collected either rectally or non-invasively promptly after defecation, and were immediately mixed with 2.5% potassium dichromate and stored at 4°C before transportation to the Parasitology Department, Norwegian University of Life Sciences (NMBU) for analysis. The ages of the goats were not recorded, but the majority were adults.

Figure 1: Areas where samples were collected.

Analysis of goat faeces for *Cryptosporidium* oocysts and *Giardia* cysts

The samples were analysed by immunomagnetic separation (IMS) and direct faecal smears with immunofluorescent antibody staining for the presence of *Giardia* cysts and/ or *Cryptosporidium* oocysts.

IMS analysis

Faecal samples were washed with phosphate buffered saline, and then passed through a faecal parasite concentrator with a pore diameter 425 µm (Midi Parasep, Apacor, Berkshire, England) and centrifuged to create a pellet. *Giardia* cysts and *Cryptosporidium* oocysts were isolated using an in-house immunomagnetic separation method (IMS) using Dynabeads®(GC-Combo, Life Technologies, Carlsbad, CA) [28, 29]; 10 µL anti-*Giardia* beads, 10 µL anti-*Cryptosporidium* beads, 80 µL Sur-Modics StabilZyme® , 20 µL SL Buffer B and 100µL Buffer Q4 were used to generate 55 µL of purified sample from approximately 200 mg of the faecal pellet. 5 µL of the resulting purified sample was dried and fixed with methanol to multispot welled slides for detection of *Giardia* cysts and *Cryptosporidium* oocysts using a *Cryptosporidium/Giardia* direct immunofluorescent antibody test (IFAT; Aqua-Glo, Waterborne Inc., New Orleans), in accordance with manufacturer's instructions.

Prior to being screened, samples were also stained with 4'6 diamidino-2-phenylindole (DAPI), a non-specific fluorescent stain that binds to double-stranded DNA.

Faecal smears

Between 5-20 µL of homogenized and sieved faecal material was placed on a microscope slide using plastic bacteriological loops that take approx. 10µL amount of sample. The samples were left to dry and then fixed with methanol before staining with 15 µL of monoclonal antibody and incubation as described for IMS. DAPI staining was not used in this preparation due to the amount of other DNA – containing debris in faecal smears.

After fixing and staining, preparations from both faecal smears and IMS were screened under a fluorescent microscope with the following filter settings: FITC: emission- 490, excitation – 525 and DAPI: emission- 350, excitation – 470.

The samples were graded after counting the number of cysts/oocysts per field of view at x20 magnification:

Table 1: Grading of cyst counts visualized using immunofluorescent microscopy

Cyst count	Grading
1-9	+
10 – 50	++
>51	+++
>100	++++

Molecular methods

DNA extraction

Each microcentrifuge tube containing *Cryptosporidium* oocysts and *Giardia* cysts were re-suspended

in Tris-EDTA buffer and held at 100°C for *Cryptosporidium* oocysts and 90°C for *Giardia* cysts for one hour, before the DNA was isolated using QIamp DNA mini kit (Qiagen GmbH), using an overnight step at 56°C.

PCR, electrophoresis, purification of PCR product, and sequencing

Samples that were DAPI – positive were selected for genotyping and PCR, regardless of number of (oo)cysts.

Four genes were used for genotyping investigations of the *Giardia* positive samples by conventional PCR; the β-giardin gene, the glutamate dehydrogenase (*gdh*) gene, the triosphosphate isomerase (*tpi*) gene, and the small subunit ribosomal RNA (SSU RNA). For the sample with *Cryptosporidium* oocysts, primers targeting sections of the genes SSU, COWP, and Actin were used, also by conventional PCR. The primers and reaction cycles are further described in the supplementary table.

For all genes, the following PCR mixture was used: 10 pmol of each primer, 0.4 µl of bovine serum albumin (20 mg/mL), 5.8 µl of water, 25 µl of HotStartTaqmaster (QIAGEN® GmbH, Germany), and 2 µl of template. For each set of reactions, a negative control (water) and a positive control (DNA from *G. duodenalis* H3 isolate belonging to assemblage B, Waterborne Inc., New Orleans, USA, and *Cryptosporidium parvum* oocysts, with species identification by Hønsvall and Robertson [30]) were included.

PCR products were electrophoresed on 1% agarose gels and stained with SYBRsafe® DNA gel stain under UV radiation. Positive samples were purified using ROCHE® high pure PCR product purification kit, and purified products were sent along with appropriate primers for sequencing on both strands at GATC biotech, Germany. Sequences were examined using Geneious 10.1.2 software and sequence comparisons conducted using NCBI BLAST.

Sequences were submitted to GenBank and their Accession numbers are provided in the results.

Statistics

The two methods, IMS and smear, were analysed using 40 randomly selected samples and compared using Fisher's exact test, based on categorical data in a two-by-two contingency table.

For comparison of *Giardia* prevalence according to location (urban/peri-urban), Students T-test was used. Similar comparisons for *Cryptosporidium* were not conducted, due to low prevalence.

Results

Comparison of faecal smears and IMS for detection of oocysts in goat faecal samples

A comparison of the two faecal examination methods is shown in a bar chart (Figure 2). Using IMS detected significantly more positive samples than using a smear ($P < 0.001$; Table 2).

Table 2: Contingency table, results of Fisher's exact test.

		Smear		
		Positive	Negative	Total
IMS	Positive	12	13	25
	Negative	0	15	15
	Total	12	28	n = 40
	Values			
	p-value			0.001

Figure 2: Bar plot. Recovery and identification by IMS and faecal smear.

Prevalence of *Cryptosporidium* and *Giardia* in goat samples

The samples were taken from flocks with a majority of adult animals. The faecal samples were firm and pelleted, and there were no signs of diarrhoea in the samples.

Examination of the faecal samples using immunofluorescent microscopy revealed the presence of *Giardia* cysts in 34.3% (71/207) of samples and *Cryptosporidium* in 0.5% of samples (Table 3).

The prevalence in the urban areas was 30.5% (32/105), and the prevalence in the rural areas was 37.3% (38/102). These means were not statistically different ($P > 0.05$).

Table 3: Overall prevalence according to area of sampling

Place	No of samples	*Giardia* positive	*Cryptosporidium* positive
Chandigarh			
Kansal	71	13	ND
Sector 38 West	30	18	ND
Maloya	4	1	ND
Punjab			
Jayenti	29	9	ND
Singha Devi	10	1	1
Kurali	20	10	ND
Haryana			
Saketri	43	18	ND
Total	207	71	1

ND: not detected.

Intensity of shedding of *Cryptosporidium* and *Giardia* in goat samples

Of the *Giardia*-positive samples, most (75%) had a low to moderate (+ and ++) number of cysts, and 25% had a high number of cysts (+++ and ++++) (Table 4). Goats excreted 55 to over 55000 cysts per gram faeces (Mean: 8671, Median: 275).

The *Cryptosporidium* positive samples had moderate (++) oocyst excretion (Table 4).

Table 4: Intensity of infection from positive samples and sampling area according to IMS results

Intensity of infection	G+	G++	G+++	G++++	C++
Chandigarh					
Kansal	10	2	-	-	-
Sector 38 West	8	2	1	8	-
Maloya	1	-	-	-	-
Punjab					
Jayenti	4	3	-	-	-
Singha Devi	1	-	-	-	1
Kurali	5	-	-	5	-
Haryana					
Saketri	11	6	-	4	-
Total	40	13	1	17	1

- : not detected

Molecular analyses

The PCR at different genetic loci had the following sensitivities: SSU 50% (26/52), Beta-giardin 1.9% (1/52) TPI 5.7% (3/52) GDH 9.6% (5/52).

228 PCR and sequencing on the single *Cryptosporidium*-positive sample revealed *C. ubiquitum* (GenBank
229 Accession number: MF124820)
230
231 An overview of the *Giardia* genotyping results is provided in Table 5. Based on all the results
232 combined from the different PCR, the majority (68 %) of *Giardia* Assemblages identified were
233 potentially zoonotic (A or B), with 10 out of 28 (36 %) genotyped samples Assemblage A, 9 (32 %)
234 Assemblage B, 8 (29 %) Assemblage E, 1 and one (4 %) Assemblage D. One of the samples was
235 sequenced to be Assemblage E at the GDH gene, and Assemblage C at the SSU gene.
236
237 **Table 5:**

238 Discussion

239 The main finding of this cross-sectional survey is that although *Cryptosporidium* prevalence was
240 relatively low in the goats included, the prevalence of *Giardia* excretion was relatively high, and with
241 a preponderance of potentially zoonotic Assemblages, indicating that goats may be both a source for
242 human infection, and also may themselves be infected by *Giardia* excreted from humans.

243 Studies from Belgium[31], Netherlands [32], Spain [33, 34] Iran [35] and Uganda [36] have revealed
244 prevalences of *Giardia* in goats ranging between from 12.3% to 42.2%, with the lowest prevalence
245 found in Uganda.

246 For *Cryptosporidium*, the prevalences in different studies from Belgium, Brazil, Sri Lanka, France and
247 Spain were in ranges varying from 4.8% to 33.6% [31, 34, 37-39].

248 Reported prevalences for both *Giardia* and *Cryptosporidium* in goats around the globe tend to vary
249 considerably. This might not only reflect the prevalence of infection, but could also be due to the
250 sensitivity of the diagnostic test used, the age of the goat, and whether only a single or consecutive

sample(s) was taken, given the intermittent shedding of *Giardia* cysts, and the acute nature of cryptosporidiosis. The low prevalence of *Cryptosporidium* in our study probably reflects that most of the samples were derived from adult animals.

When determining whether animal giardiasis and cryptosporidiosis is of relevance in a public health context, it is imperative to identify the species and genotypes involved. The SSU rRNA marker, which had the highest sensitivity in this study, is commonly used for assemblage differentiation of *Giardia duodenalis* assemblages, though the amplicon is small and might be insufficient for confident identification of the assemblage due to low levels of phylogenetic resolution, perhaps related to its multi-copy nature [40]. However, although only a few samples provided genotyping results at more than one gene, in these cases the results were consistent between the different loci. In addition, infections with mixed assemblages are quite common in ruminants, especially those involving assemblage A and E [40]. To improve the knowledge of endemicity of *Giardia* assemblages in specific study areas, especially where zoonotic or anthroponotic infections are implied, primers specific for assemblages should be further developed, as it could be imperative in source tracking and identification[40].

Although Assemblage E is generally considered to infect only hoofed animals, infections with Assemblage E have been recently reported from humans in Egypt [41] and Brazil [42]. Finding *Giardia* from Assemblage B in goats is rather unusual compared to other studies; a review from 2009 reports that Assemblage E is most frequently reported – with potentially zoonotic infection, particularly with Assemblage B, occurring relatively rarely [21]. The difference between our study and many of the other studies is the close contact in our study between the goats being sampled and the human environment. The proximity of humans and goats in our study area, along with the supporting evidence for molecular results, might suggest zoonotic /anthropozoonotic spread of the parasite in such situations.

275 One sample was sequenced as assemblage D, which is a canid-specific genotype. As this sample had low numbers of cysts it seems likely that this represents carriage from the goat ingesting cysts from dog faeces and then excreting them, rather than infection. This may also be the case where Assemblage E was found at one gene, and Assemblage C at another. Whether this may apply to other samples cannot be determined.

280 Goats are not picky eaters, however, their grazing habits are generally more similar to that of deer as they are browsers, preferring woody shrubs and weeds rather than grass. This usually implies that they are less likely to ingest parasites, but in an urban or peri-urban setting where shrubs are scant, they will be forced to search for nutrients closer to the ground, thus being more likely to ingest *Giardia* cyst or *Cryptosporidium* oocysts excreted by a human or another animal, and either getting infected themselves or carry the parasite until it is excreted again.

286 One of the samples contained *Cryptosporidium ubiquitum* that perhaps infects the greatest number of host species of all *Cryptosporidium* spp. This species has been found in a wide range of domestic and wild animals as well as humans [43], and thus represents a considerable zoonotic and anthroponotic potential, especially in the setting of a shared household between goats and humans in lower socioeconomic areas. In addition, with *Cryptosporidium*, there is a vast amount of epidemiological data demonstrating strong links between contact with infected livestock and human infections [1].

293 As most of these goats were living close to humans and often sharing the same household, it is not unlikely that they might share some intestinal parasites as well.

295 There was no significant difference between the samples collected in urban and peri-urban areas, and the genotyping results showed an even spread of the *Giardia* genotypes in the given areas. Goat husbandry in India is usually a subsidiary activity to supplement income. It is generally based on a free-ranging management, allowing them to graze in open fields and wastelands, as goats have the economic advantages of not requiring expensive housing and feed. This makes them especially

attractive to human populations with limited resources and thus also limitations from clean water, proper sewage, dung and garbage disposals making the hygiene of the husbandry prone to contamination of parasites from both humans to animals and vice versa.

As only one faecal sample was collected per animal, the prevalence data may underestimate the actual number of infected animals due to the intermittent shedding of cysts. Also, *G. duodenalis* infections usually last longer than *Cryptosporidium* infections, often becoming chronic, whereas cryptosporidiosis may be acute, but usually with spontaneous recovery over a relatively short time period. Thus, *Giardia* cysts are more likely to be identified in single samples [34], which may partly explain the low prevalence of *Cryptosporidium* oocysts in this study.

Although using IMS for analysis of faecal samples is more time-consuming and expensive our results indicate that it provided more positive results; this is presumably due to the larger quantity of faecal matter than can be analysed and also, perhaps, due to less debris in the sample. In cleaner samples it is also easier to determine the suitability of the sample for molecular analyses based on DAPI-staining due to there being lower background fluorescence.

This method could be a useful tool for other field studies where it is only possible to obtain one sample per animal, not the three consecutive ones which are recommended due to the intermittent shedding of cysts to obtain a more certain answer of the true prevalence.

Infected livestock have long been suggested as sources for contaminating food and water in outbreaks, but molecular analyses has often incriminated human effluent as the source[1]. Nonetheless, as an adult goat produces between 1-3 kg of faeces on a daily basis, it is clear that the potential for environmental contamination is considerable [21], especially when the animals are kept on a free-range basis in a community where the overall density is high. The common characteristics of *Giardia* and *Cryptosporidium*, having a low infectious dose, (oo)cysts being infective immediately after excretion, and their robustness enabling them to survive for months in the environment [44],

324 are epidemiological traits well-suited for causing infectious foci in places with high population

325 densities and extensive animal husbandry.

326 In addition, a serious constraint to economical and intensive goat production is the mortality of kids

327 as a result of diarrhoea up to the age of three months [45], and among the pathogens causing the

328 diarrhoea *Cryptosporidium* is principally involved [38, 46]. *Giardia* infection in ruminants is, on the

329 other hand, often asymptomatic, but may also be associated with the occurrence of diarrhoea and ill-

330 thrift [1], which may lead to economic losses as well as reduced welfare of the flock.

331 Within the global distribution of goat populations, there are very few available publications that

332 report on these infections in places where they are most likely to exert the greatest impact on the

333 human population [21]and vice versa.

334 **Conclusion**

335 As keeping goats in developing countries is usually a trade for the poorest in society, the awareness

336 of One Health for one household through proper hygienic routines and animal management could be

337 of benefit for both human and animal health, as well as improving both the economy and husbandry

338 of the goatkeepers and their herds.

339

340 **Declarations**

341 **Ethics approval**

342 As the sample collection was non-invasive and did not involve handling any animals, ethical

343 approval was not sought.

344 **Authors Contribution**

345 KSU planned the study, collected the samples and was in charge of handling, analysis and

346 interpretation of the results, and was the main writer of this article.

NM analysed a substantial amount of the samples, was in charge of comparing the results of IMS/fecal smear analysis, and was a major contributor in writing the manuscript.

RSB facilitated the organizing of the sampling process, and contributed in writing the manuscript.

HJ and AK made collection of the samples possible, as well as contributing in writing the manuscript.

LJR was the main driver of putting this article together, and contributed substantially to the outlay of the manuscript, statistical analysis and methods applied in this study.

All authors read and approved the final manuscript

Consent for publication

Not applicable

Availability of data

The sequences found in this study are available at the U.S National Library of Medicine, and accession numbers can be found in the database NCBI BLAST available at https://blast.ncbi.nlm.nih.gov/Blast.cgi

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Conflict of interest

The authors state that they have no competing interests.

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Table 5: Results from sequencing and analysis of positive PCR products. *Giardia* assemblages are noted with capital letter.

Area	Sample no	No. of cysts isolated by IMS[a]	DAPI[b]	GDH[c]	TPI[d]	Bg[e]	SSU[f]
Chandigarh							
Kansal	1	10	100%	-	-	-	B MF069062
	2	220	30 %	-	-	-	E MF069058
	3	10	100%	-	-	-	A MF069057
	4	20	100 %	-	-	-	B MF069047
	5	100	40 %	-	-	-	A MF069052

	6	10	100%	-	-	-	A MF069051
Sector 38 West							
	7	1000	70 %	E MF084938	-	-	C MF069071
	8	10000	5 %	E MF084935	-	-	E MF069070
	9	10	100%	-	-	-	A MF069056
	10	10000	90 %	-	-	-	E MF069059
	11	100	60%	-	-	-	D MF069055
Punjab							
Singha Devi							
	12	30	67 %	-	-	-	A MF069054
Kurali							
	13	10000	90 %	E MF084936	E MF095054	E MF106203	E MF069072
	14	10000	70 %	E MF084934	E MF095052	-	-
	15	10000	80 %	-	B MF095053	-	B MF069053
	16	20	100%	-	-	-	B MF069066
	17	20	50%	-	-	-	B MF069064
Jayenti							
	18	10	100%	-	-	-	B MF069060
Haryana							
Saketri							
	19	100	10%	E MF084937	-	-	-

20	50	60%	-	-	-	B MF069050
21	30	30%	-	-	-	A MF069068
22	100	40%	-	-	-	A MF069067
23	1000	90 %	-	-	-	E MF069065
24	40	25 %	-	-	-	B MF069063
25	20	50 %	-	-	-	B MF069061
26	120	33%	-	-	-	A MF069069
27	500	44%	-	-	-	A MF069049
28	160	87 %	-	-	-	A MF069048

- : no amplification

TPI, Triosephosphate isomerase; GDH, glutamate dehydrogenase; BG, beta giardin; SSU, small subunit rRNA; -, PCR Negative; Assemblage (GenBank Accession number) where sequence of PCR products was obtained.

[a] Number of Giardia cysts used for DNA isolation

[b] proportion of DAPI positive *Giardia* cysts used for DNA isolation

[c] Read et al. (2004)

[d] Sulaiman et al. (2003)

[e] Lalle et al. (2005)

[f] Hopkins et al. (1997) & Read et al. (2002).

PCR conditions and reaction times can be found in Additional file 1.

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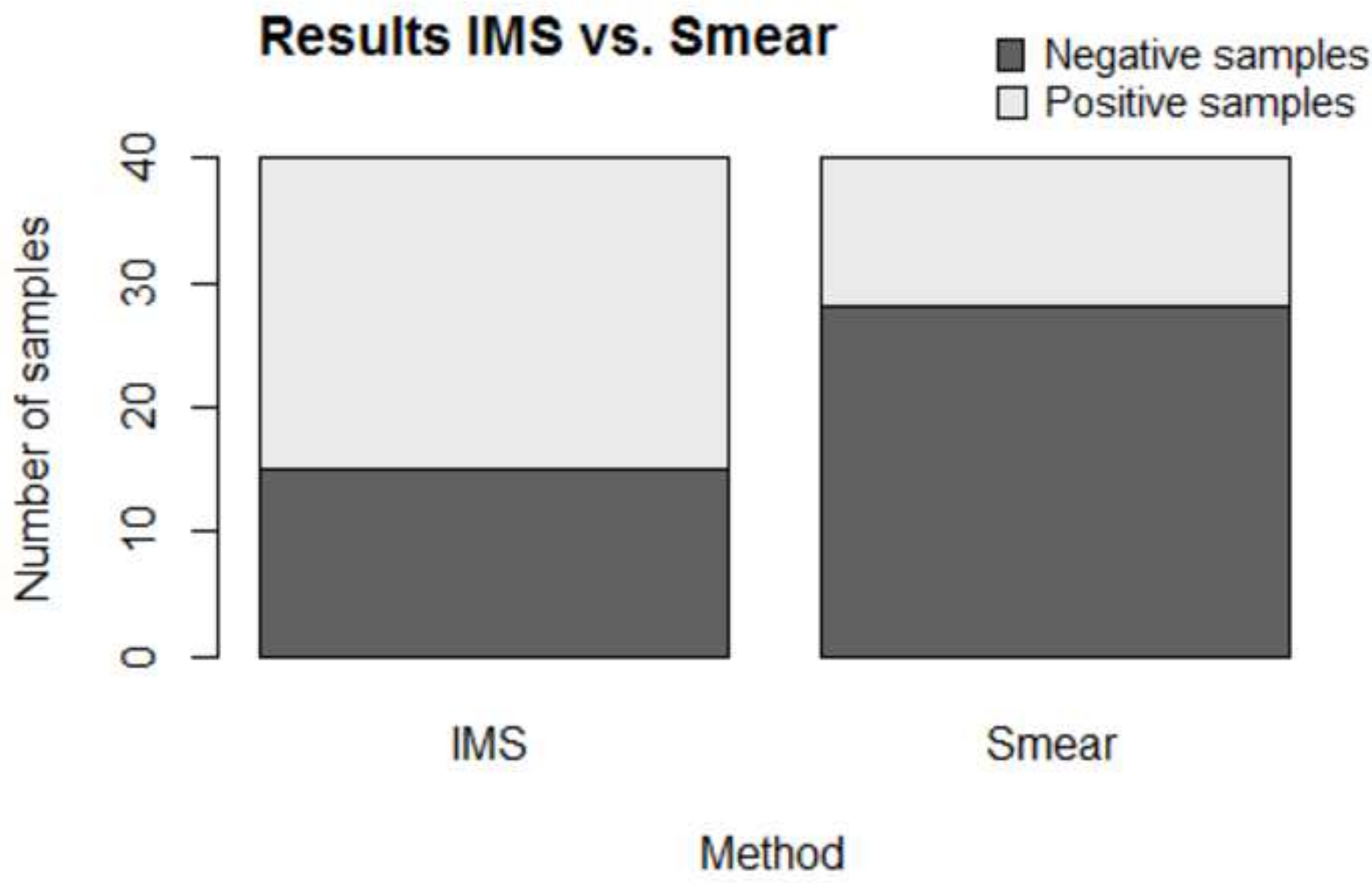
Figure Click here to download Figure Fig. 2.tif



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Supplementary table 1. PCR conditions for detection of *Giardia* and *Cryptosporidium.*

Locus	Amplicon length	Primer	Cycle conditions		References
Giardia					
Small Subunit (SSU) rRNA	292	1st amplification	96 °C, 5 min		[1]
		F: 5′-CATCCGGTCGATCCTGC-3′	96 °C, 30 sec	40 x	[2]
		R: 5′-AGTCGAACCCTGATTCTCCGCCAGG-3′	59 °C, 40 sec		
			72 °C, 40 sec		
			72 °C, 7 min		
	175	2nd amplification	96 °C, 5 min		
		F: 5′-GACGCTCTCCCCAAGGAC-3′	96 °C, 30 sec	50 x	
		R: 5′-CTGCGTCACGCTGCTCG-3′	55 °C, 40 sec		
			72 °C, 30 sec		
			72 °C, 7 min		
Triosephosphate Isomerase (TPI)	605	1st amplification	95 °C, 10 min		[3]
		F: 5′-AAATYATGCCTGCTCGTCG-3′	94 °C, 45 sec	45 x	
		R: 5′-CAAACCTTYTCCGCAAACC-3′	50 °C, 45 sec		
			72 °C, 60 sec		
			72 °C, 10 min		
	563	2nd amplification	95 °C, 10 min		
		F: 5′-CCCTTCATCGGNGGTAACTT-3′	94 °C, 45 sec	45 x	
		R: 5′-GTGGCCACCACVCCCGTGCC-3′	50 °C, 45 sec		

			72 °C, 60 sec		
			72 °C, 10 min		
Glutamate Dehydrogenase (GDH)		1st amplification:	94 °C, 15 min		[4]
		F: 5′-TCAACGTYAAYCGYGGYTTCCGT-3′	94 °C, 45 sec		[5]
		R: 5′-GTTRTCCTTGCACATCTCC-3′	54 °C, 45 sec	50 x	
			72 °C, 45 sec		
			72 °C, 10 min		
Beta Giardin (BG)	753	1st amplification:	95 °C, 15 min		[6]
		F: 5′-AAGCCCGACGACCTCACCCGCAGTGC-3′	94 °C, 30 sec		[7]
		R: 5′-GAGGCCGCCCTGGATCTTCGAGAC GAC-3′	60 °C, 30 sec	35 x	
			72 °C, 60 sec		
			72 °C, 10 min		
	511	2nd amplification	95 °C, 15 min		
		F: 5′-GAACGAGATCGAGGTCCG-3′	95 °C, 30 sec		
		R: 5′-CTCGACGAGCTTCGTGTT-3′	53 °C, 30 sec	40 x	
			72 °C, 60 sec		
			72 °C, 10 min		
Cryptosporidium					
SSU rRNA	860	1st amplification	95 °C, 15 min		[8]
		5′-GGAAGGGTTGTATTTATTAGATAAAG-3′	94 °C, 45 sec		
		5′-AAGGAGTAAGGAACAACCTCC A-3′	55 °C, 45 sec	50 x	
			72 °C, 60 sec		
			72 °C, 10 min		

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VI

Manuscript Details

Manuscript number	VPRSR_2017_201
Title	Prevalence and zoonotic potential of intestinal protozoans in bovines in Northern India
Article type	Full Length Article

Abstract

Bovines, and especially cattle, have a dual position of appreciation in India, being both important in the food industry as providers of dairy products, and, culturally, being considered as holy creatures that it is forbidden by law to harm, kill, or eat. This status means that cattle have a paradoxical existence in India; as they are worshipped and protected, they are able to roam freely amongst humans, but they are also often left to fend for themselves. The vast numbers of roaming cattle without clear owners are difficult to look after in terms of veterinary healthcare and appropriate interventions when necessary, and have no regular supply of food. This article describes an investigation of the occurrence of Cryptosporidium spp. and Giardia duodenalis in bovines either roaming the streets or being kept in animal holdings in and around Chandigarh, a city in Northern India, and addresses the zoonotic potential of these protozoan parasites shed from bovines living in close contact with humans. Animals of all ages were sampled, and the majority of the positive samples were found from calves. The overall prevalence of Giardia was 8.2% and Cryptosporidium was 2.4%. Non-zoonotic assemblages were predominantly found in the case of the Giardia – positive samples, and in the case of Cryptosporidium, as well as non-zoonotic genotypes, zoonotic subgroups previously described from infected human infections in this area, were identified, indicating that there may be sharing of intestinal parasites in these settings, where cattle live in close connection to humans.

Keywords	Bovines; Cryptosporidium; Giardia; zoonosis; India
Taxonomy	Cattle, Cryptosporidium, Giardia, Public Health, India, Zoonoses

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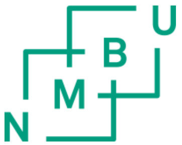

11th September 2017

Dear Editor

Submission of Manuscript: Prevalence and zoonotic potential of intestinal protozoans in bovines in Northern India

Please find attached a manuscript that we would like to have considered for publication in Veterinary Parasitology – Regional Studies and Reports.

In brief, the research described in the manuscript investigates the prevalence and zoonotic potential of *Cryptosporidium* oocysts and *Giardia* cysts in feces from bovines sampled over a longer period from different areas in and around Chandigarh, a city in Northern India.

The results indicate that there is a relatively low occurrence of protozoans in bovines in Chandigarh, with some isolates having zoonotic potential.

All authors have agreed to the submission of this version of the manuscript.

Best regards

Kjersti Selstad Utaaker

- In India, cattle are both worshipped and neglected
- These bovines live in close contact with humans under poor hygienic settings
- The close interface may break down barriers of diseases transmitted between them
- This article investigates the occurrence of zoonotic protozoans in Indian bovines

1 **Prevalence and zoonotic potential of intestinal protozoans in bovines in Northern India**

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12

13 **Abstract:**

14 Bovines, and especially cattle, have a dual position of appreciation in India, being both important in
15 the food industry as providers of dairy products, and, culturally, being considered as holy creatures
16 that it is forbidden by law to harm, kill, or eat. This status means that cattle have a paradoxical
17 existence in India; as they are worshipped and protected, they are able to roam freely amongst
18 humans, but they are also often left to fend for themselves. The vast numbers of roaming cattle
19 without clear owners are difficult to look after in terms of veterinary healthcare and appropriate
20 interventions when necessary, and have no regular supply of food.

21 This article describes an investigation of the occurrence of *Cryptosporidium* spp. and *Giardia*
22 *duodenalis* in bovines either roaming the streets or being kept in animal holdings in and around
23 Chandigarh, a city in Northern India, and addresses the zoonotic potential of these protozoan
24 parasites shed from bovines living in close contact with humans. Animals of all ages were sampled,
25 and the majority of the positive samples were found from calves. The overall prevalence of *Giardia*
26 was 8.2% and *Cryptosporidium* was 2.4%. Non-zoonotic assemblages were predominantly found in
27 the case of the *Giardia* – positive samples, and in the case of *Cryptosporidium*, as well as non-
28 zoonotic genotypes, zoonotic subgroups previously described from infected human infections in this
29 area, were identified, indicating that there may be sharing of intestinal parasites in these settings,
30 where cattle live in close connection to humans.

31

32 **Key words:** Bovines, *Cryptosporidium*, *Giardia*, zoonosis, India

33

34 **1. Introduction**

35 *Cryptosporidium* and *Giardia* are two of the most common infectious agents of infectious enteritis in
36 humans and animals worldwide. From a public health perspective, it is imperative to understand the
37 sources and routes of transmission in different geographical regions. *Cryptosporidium* and *Giardia*
38 infections are known to cause production losses in bovines, and are considered potential sources of
39 human infection as well, with pre-weaned calves and lambs recognised as important reservoirs of the
40 zoonotic *C. parvum* in some countries (Santin-Duran and Trout, 2008).

41 The extent and relative importance of zoonotic transmission of these parasites in different parts of
42 the world, especially in the developing countries, are still poorly understood (Abeywardena et al.,
43 2015). Although for *Giardia*, it is generally considered that most human infections are from direct or
44 indirect human-to-human transmission, rather than zoonotic (Monis and Thompson, 2003), for
45 *Cryptosporidium* a different picture occurs.

46 In human populations, it is generally considered that *Cryptosporidium parvum* tends to dominate in
47 Europe, New Zealand, and the Middle East, indicating a potential for zoonotic transmission, whereas
48 *C. hominis* infections are responsible for more human infections than *C. parvum* in USA, China, Japan,
49 and the majority of developing countries (Cacciò and Putignani, 2014).

50 Indeed, studies in India tend to support this distribution; three studies from Northern India indicated
51 that over 70 % of *Cryptosporidium* infections were caused by *C. hominis* (Yadav et al, 2017)(Gatei et
52 al., 2007; Sharma et al., 2013), which is in concordance with the general finding of Xiao and Fayer
53 (2008), who reported that, in the studies where molecular methods have been used, *C. hominis* has,
54 in general, been associated with a higher number of human infections than *C. parvum*.

55 However, despite this distribution, the role of cattle in transmission or propagation of these parasites
56 in India is of particular interest, due to the particular interactions between bovids and humans here.
57 India is home to a quarter of the world's cattle population. In Hindu religious scriptures, the cow is
58 referred to as the "all-producing and all-containing universe"(Korom, 2000), and the cow is the most

59 sacred of all animals according to the Hindu religion. Northern India practices a total ban on cow
60 slaughter, and the Indian Veterinary Council has estimated that there is only sufficient food to
61 sustain 60% of the Indian cattle population, and the rest are left to starve or roam the streets
62 searching for food, which, in most cases, ends up being a diet of garbage (Agoramoorthy and Hsu,
63 2012; Kang, 2003). In New Delhi, there is an estimated number of 40 000 stray cattle, and in
64 Chandigarh this number has risen from 1400 to 2000 during the last five years (Victor, 2013). This
65 creates an obvious difference in animal management from industrialized countries, where cattle are
66 kept on enclosed farms and restrictions between animal and human contact are mandatory by law
67 due to zoonosis and hygiene considerations. The situation in India also differs from that in other
68 developing countries, where cow ownership and cattle as a staple means of livelihood means that
69 cattle are pastured as far as possible to ensure that their productivity as dairy or beef animals is
70 maximised, and unrestricted wandering, particularly in urban settings, is relatively uncommon. The
71 special role of cows in Indian culture means that the interface between human and animals is
72 minimal, and the sources of infection, for both human and animals, are likely to be strolling along, or
73 sometimes scavenging for food, in the same streets.

74 In this study, faecal samples from cattle and water buffaloes in urban and peri-urban areas of
75 Northern India were collected, and the prevalence and zoonotic potential of *Cryptosporidium* and
76 *Giardia* were assessed.

77 **2. Material and Methods**

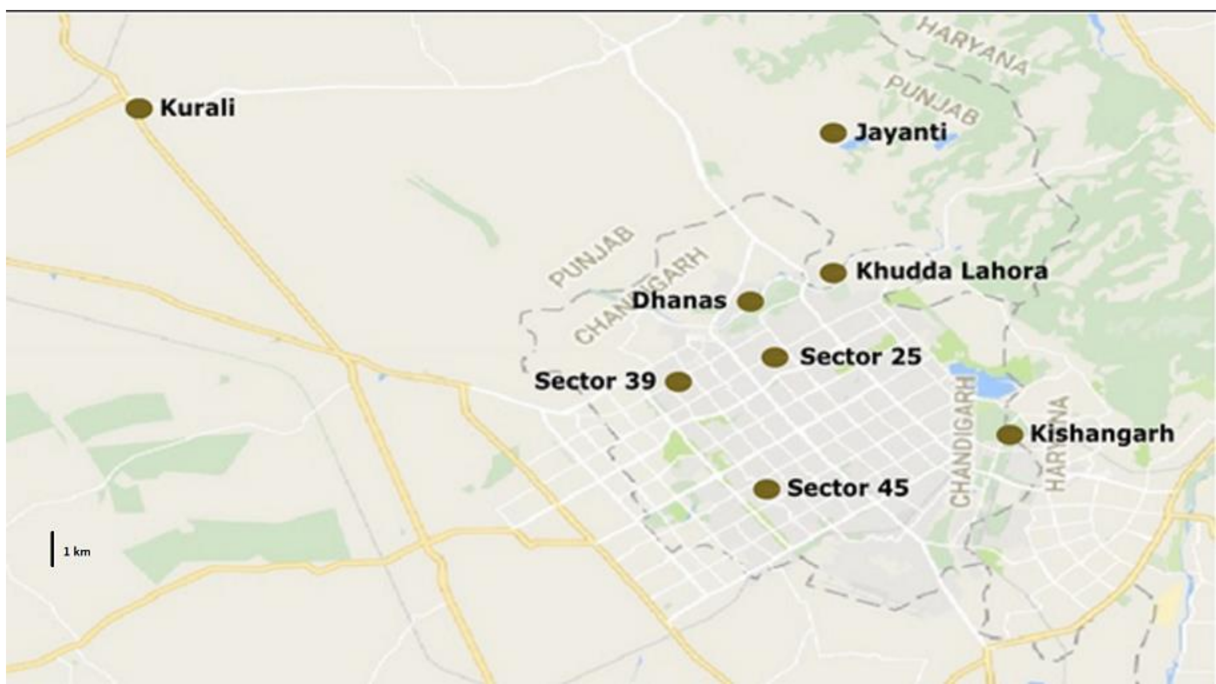
78 **2.1. Sampling**

79 From March 2014 until February 2016, 294 samples were collected from different animal holdings in
80 Chandigarh, Northern India. Of these, seasonally, 109 samples were collected during the winter, and
81 185 during the monsoon season. With respect to where the various bovines lived, 153 samples were
82 collected from animal holdings within the city (urban), and 141 from animal holdings and settlements
83 surrounding the city (peri-urban). Only one sample was collected per animal per sampling occasion.

84 The ages of the animals ranged from calves under three months to adults. Presence of diarrhoea in
85 samples and approximate age of animal was recorded at sampling. The samples (each approximately
86 5-10 g) were collected either rectally or non-invasively promptly after defecation, and were
87 immediately mixed with 2.5% potassium dichromate and stored at 4°C before transportation to the
88 Parasitology Department, Norwegian University of Life Sciences (NMBU) for analysis.

89

90 **Figure 1**: Map of sampling sites



91

92 **2.2. Analysis for occurrence**

93 From each sample collected, 3 grams of faeces were homogenized with 57 mL water and passed
94 through a faecal parasite concentrator with a pore diameter 425 µm (Midi Parasep, Apacor,
95 Berkshire, England). The suspension was transferred to 10 mL centrifuge tubes and centrifuged at
96 1550 rfg) for 3 minutes to create a pellet. The supernatant was discarded, and between 5-20 µl of
97 homogenized and sieved faecal material was placed on a microscope slide using plastic
98 bacteriological loops that take approx. 10µl amount of sample. The samples were left to dry and then
99 fixed with methanol before staining with 15 µl of monoclonal antibody for *Cryptosporidium* oocysts
100 and *Giardia* cysts (AquaGlo etc), and then incubated at 37°C in a humid chamber for 45 minutes. The
101 staining solution was then rinsed off with distilled water, and a coverslip placed over the sample
102 before immediate microscopic examination.

103 Prepared samples were screened under a fluorescence microscope with the following filter settings:
104 FITC: emission- 490, excitation – 525.

105 The samples were graded after counting the number of cysts/oocysts per field of view at x20
106 objective magnification:

107 **Table 1:** Grading of (oo)cyst counts by immunofluorescent microscopy

Oocyst / Cyst count	Grading
1-9	+
10 – 50	++
>51	+++
>100	++++

108

109 Positive samples were further examined at x40 objective to confirm presence of (oo)cysts.

110 **2.3. DNA isolation**

111 For *Giardia*/*Cryptosporidium*-positive samples, DNA was isolated from 200 µg of concentrated faeces
112 using the QIAmp DNA mini kit (Qiagen GmbH). The protocols followed the manufacturers`
113 instructions with slight modifications; cysts/oocysts were first mixed with 150 µl of TE buffer
114 (100 mM Tris and 100 mM EDTA) and incubated at 90 °C/100 °C (*Giardia*/*Cryptosporidium*) for 1 h
115 before an overnight proteinase K lysis step at 56 °C and spin column purification. DNA was finally
116 eluted in 30 µl of PCR grade water and stored at 4 °C prior to PCR.

117 **2.4. PCR and sequencing**

118 In all cases, the primary PCR consisted of 8.3 µl PCR water, 1 µl forward and 1 µl reverse primer (at a
119 final concentration of 0. mM), 0.2 µl BSA (20 mg/l), 12.5 µl of 2× HotStartTaqMaster and 2 µl of
120 template DNA. For each PCR, positive and negative controls were included. PCR products were
121 visualized by electrophoresis on 2% agarose gel with Sybr Safe stain (Life Technologies, Carlsbad, CA).
122 Target genes and PCR conditions are provided in Supplementary table X.

123 When positive results were obtained, the DNA amplicons were purified using ExoSAP-IT® cleanup kit
124 from Affymetrix USB and sent to GATC Biotech, Germany for sequencing. Sequences were examined
125 using Geneious 10.1.2 software and sequence comparisons conducted using NCBI BLAST. Sequences
126 were submitted to GenBank and have been assigned Accession numbers given in table 2. Sequences
127 from both directions were assembled and manually corrected by analysis of the chromatograms
128 using the program Geneious™.

129 **2.4.1. *Giardia***

130 Conventional PCR was performed on *Giardia*-positive samples targeting the small subunit rRNA (SSU
131 rRNA) and the triosephosphate isomerase (TPI) genes, following the protocols of Hopkins et al.
132 (1997) and Sulaiman et al. (2003).

133 **2.4.2. *Cryptosporidium***

134 Conventional PCR was performed on *Cryptosporidium* positive samples at the SSU rRNA gene
135 according to the protocol of Xiao et. al. (1999), and the Gp60 gene according to Glaberman et al
136 (2002).

137 **2.5 Statistical analysis**

138 A database of the results was created in Microsoft Excel. Associations of excretion of
139 *Cryptosporidium* oocysts and /or *Giardia* cysts with animal or sampling characteristics were explored
140 by contingency table analysis.

141

142 **3. Results**

143 **3.1. Occurrence**

144 Overall, 28 (9.5%) of the samples were positive for either *Giardia* cysts or *Cryptosporidium* oocysts
145 (8.2% of the samples were *Giardia* positive, and 2.4% were *Cryptosporidium* positive), and 3 samples
146 were positive for both parasites; see Table 2.

147 **3.2 Intensity of cyst/oocyst excretion by age and other sampling characteristics**

148 Of the *Cryptosporidium*-positive samples, all were from calves, and oocyst excretion was either ++ or
149 +++ (no low shedders detected).

150 Although none of the adult cows were shedding *Cryptosporidium* oocysts, low level excretion of
151 *Giardia* cysts was detected in the faeces of 5 adult cows. Calves were found to be both low level
152 excretors of *Giardia* cysts (47% of those shedding *Giardia* cysts were recorded as +), and high level
153 excretors (35% recorded as +++).

154 Those calves excreting *Cryptosporidium* oocysts often had diarrhoea, with the consistency of the
155 faeces varying from firm to diarrhoeic, whereas diarrhoea was not common in those excreting
156 *Giardia* cysts. However, the data were not consistently recorded and therefore statistical associations
157 could not be explored.

158 Time of year of sample collection (winter or monsoon seasons) was not associated with increased
159 prevalence, and neither was sampling location.

160

161 **3.3 Molecular results**

162 Only the samples from calves had sufficient (oo)cysts to be considered suitable for molecular
163 investigations, and this was not successful in all cases. An overview of the sequencing results is
164 provided in Table 2. Among the *Cryptosporidium* samples, both *C. bovis* and *C. parvum* were
165 identified. Only one subtype of *C. parvum* was identified, IIdA15G1, in 4 samples from 3 different
166 locations. Among the *Giardia* samples, Assemblage E was found to predominate, being identified in 6
167 samples, and Assemblages A and B were identified in 1 and 2 samples, respectively.

168

169

170

171 **Table 2: Positive microscopy and sequencing results**

172

Season	Area	Result from microscopy[a]	Animal	*Cryptosporidium* SSU[b]	*Cryptosporidium* Gp60[c]	*Giardia* SSU[d]	*Giardia* TPI[e]
Winter	Urban	G+	Cow	-	-	-	-
Winter	Urban	G+	Cow	-	-	-	-
Winter	Urban	G+	Cow	-	-	-	-
Winter	Urban	C ++	Calf	*	*	-	-
Winter	Peri-urban	G+++	Calf	-	-	*	*
Winter	Peri-urban	G+	Calf	-	-	Ass B MF399205	Ass E MF459679
Winter	Peri-urban	G+	Calf	-	-	*	*
Winter	Peri-urban	C+++	Calf	*C. parvum* MF399201	*C. parvum* IIdA15G1 MF459681	-	-
Winter	Peri-urban	G+	Calf	-	-	*	*
Winter	Peri-urban	G+	Calf	-	-	Ass A MF163432	*
Winter	Peri-urban	C+++	Calf	*C. bovis* MF399200	*C. parvum* IIdA15G1 MF535626	-	-
Winter	Urban	G+++	Calf			Ass E MF399204	Ass E MF459678

Winter	Urban	G+++	Calf	-	-	*	*
Winter	Urban	G+	Calf	-	-	-	-
Winter	Urban	G+	Calf	-	-	Ass E MF163433	*
Winter	Urban	G+	Calf	-	-	*	*
Monsoon	Peri-urban	G+++	Calf	-	-	*	*
Monsoon	Peri-urban	G+	Calf	-	-	*	*
Monsoon	Peri-urban	G+	Cow	-	-	-	-
Monsoon	Peri-urban	G+	Cow	-	-	-	-
Monsoon	Urban	G+++ , C++	Calf	*	*	Ass E MF399203	*
Monsoon	Urban	C+++	Calf	*	*	-	-
Monsoon	Urban	G+++	Calf	-	-	Ass E MF399206	*
Monsoon	Urban	G+++	Calf	-	-	*	*
Monsoon	Urban	G+	Calf	-	-	*	*
Monsoon	Peri-urban	G++	Calf	-	-	*	*
Monsoon	Peri-urban	G++, C++	Calf	*C. bovis* MF399202	*C. parvum* IIdA15G1 MF535627	*	*
Monsoon	Peri-urban	G++, C++	Calf	-	*C. parvum* IIdA15G1 MF535999	*	Ass B MF459680

173

174 - : No amplification performed

175 *: Amplification was performed, but was not successful.

176 a: C = *Cryptosporidium*, G = *Giardia*; the occurrence of cysts or oocysts was scored semi-
177 quantitatively from + to +++ (see Table 1)

178 b: Species identification at SSU gene (Xiao et al, 1999)

179 c: Subtype identification at Gp60 gene (Glaberman et al, 2002).

180 d: Assemblage identification at SSU gene (Hopkins et al, 1997)

181 e: Assemblage identification at TPI gene (Sulaiman et al, 2003)

182

183 **4. Discussion**

184 The main finding from this study was that around 10 % of bovids wandering the streets of Chandigarh
185 or gathered in holdings for bovids, excrete *Cryptosporidium* oocysts and/or *Giardia* cysts. These
186 parasites were particularly prevalent in calves, and this age group also excreted higher numbers
187 (both *Giardia* and *Cryptosporidium*).

188 There was no correlation between season on shedding of (oo)cysts. Although some studies have
189 indicated seasonality of infection with *Cryptosporidium* and *Giardia* in cattle (e.g., Szonyi et al,
190 (2010); Huetink et al, (2001)are often more likely to show excretion, a study from Bangladesh found
191 no effect of season (rainy or non-rainy) on excretion of either parasite in cattle (Ehsan et al., 2015).

192

193 Chandigarh is divided into phases and sectors that differ from each other in terms of population
194 pressure and sanitary infrastructure. For simplicity, in this manuscript we have divided the sampling
195 from animals as urban (in the city, that tends to have lower human population density and better
196 infrastructure, but would not be considered appropriate for cattle in most western countries) and
197 peri-urban (generally on the edge of the city and with high population pressure, unsanitary

198 conditions, flood problems, poor garbage disposal, disposal of livestock dung into open drains, and
199 discharge of untreated sewage). Although we had anticipated that samples from bovids inhabiting
200 periurban locations would be more likely to be excreting Cryptosporidum and Giardia, our data did
201 not show this, with samples from the wealthier areas just as likely to be infected.

202 Globally, cattle are commonly infected with *C. parvum, C. andersoni, C. bovis* and *C. ryanae,* so our
203 findings are not unusual*.* *C. parvum* is most commonly found in pre – weaned calves. *C. bovis* and *C.*
204 *ryanae* in weaned calves, and *C. andersoni* in yearlings and adult cattle, and it seems mostly pre-
205 weaned calves are the major sources of *C. parvum*. Of the 14 *C. parvum* subtype families, IIa and IId
206 are the two major zoonotic subtype families in humans and animals(Wang et al., 2014). Of particular
207 interest from our study is that a single *C. parvum* zoonotic subtype (IIdA15G1) predominated in those
208 samples that were successfully sequenced, despite the positive samples being collected from
209 different locations. The subtype IIdA15G1 has also been found in cattle, sheep and goats in China,
210 Iran, Malaysia and Spain (Muhid et al., 2011; Nazemalhosseini-Mojarad et al., 2011; Quilez et al.,
211 2008; Quílez et al., 2008), and in humans in from Netherlands, Australia, India, Iran and Malaysia
212 (Ajjampur et al., 2010; Iqbal et al., 2012; Nazemalhosseini-Mojarad et al., 2011; Ng et al., 2010;
213 Wielinga et al., 2008), and the subtype family IId is considered a major zoonotic Europe, Africa,
214 Australia and Asia (Cui et al., 2014). Furthermore, this particular sub-type has previously been
215 reported from human patients with cryptosporidiosis delivering samples to a hospital in the same
216 area as where these samples were collected (Sharma, Sharma, Sehgal, Malla, & Khurana, 2013). This
217 indicates a high likelihood that cattle and humans in this area transmit *C. parvum* between each
218 other. Given the proximity of bovids and humans to each other in Indian culture, with cattle allowed
219 to wander at will and often participating in festivals, this seems not surprising. At the same time,
220 despite being worshipped, the lack of ownership means that cattle on the streets often do not have
221 access to a stable food supply, and are obliged to graze on rubbish in the ditches, which may be
222 contaminated with human faeces. Cow sanctuaries (Gaushalas) which take care of stray animals have
223 been established in many urban areas in India, and, to some extent alleviate, they do alleviate the

224 number of roaming cattle. However, their capacity is not sufficient for the rising numbers of stray
225 cattle, who often are unproductive, older cows that have been turned to the streets by their owner
226 (Singh et al., 2013). Although *Cryptosporidium* has previously been reported to be highly prevalent in
227 bovines in India, and especially among calves during the monsoon season (Hingole et al., 2017;
228 Maurya et al., 2013; Paul et al., 2008), these studies did not subtype positive samples, so the
229 transmission potential between humans and bovids could be based, at best, at the *Cryptosporidium*
230 species level.

231 Although the prevalence of *Giardia* in bovids was higher than that of *Cryptosporidium*, the majority
232 of samples were Assemblage E, suggesting that zoonotic transmission of this parasite may be less
233 likely. However, Assemblage A and Assemblage B were also detected in some samples, indicating
234 some potential for zoonotic/anthropozoonotic transmission between bovids and people in this
235 setting. Many studies on *Giardia* infections in cattle have revealed that the dominating Assemblage
236 among bovines is the species-specific Assemblage E; although generally considered not to be
237 zoonotic, some recent studies have suggested that this Assemblage may have zoonotic potential
238 (Abdel-Moein and Saeed, 2016). Assemblage A is increasingly being detected, indicating that this
239 zoonotic Assemblage may be more widespread among this livestock than assumed. A multicentre
240 trial in Germany, UK, France and Italy found an overall prevalence of *G. duodenalis* of 45.4%, and 43%
241 of those belonged to Assemblage A (Geurden et al., 2012).

242 In contrast, few studies are available regarding the zoonotic potential of *Giardia* in Indian bovines. A
243 study from West Bengal revealed a 12.2% prevalence of *Giardia*, with the majority of Assemblages
244 belonging to the species-specific Assemblage E, and 2.7% belonging to the zoonotic Assemblage A
245 (Khan et al., 2010).

246 Assemblage B is not a common finding in farm animals, and is usually associated with human
247 infection. In two of the calves sampled in this study, both *Cryptosporidium* oocysts and *Giardia* cysts
248 were found, and genotyping revealed that one calf was infected with *C. parvum* subtype IIdA15G1

249 and *G. duodenalis* Assemblage B, and this may indicate that both infections were derived originally
250 from a human source.

251 In contrast to the results from bovids described here, investigation on the occurrence of *Giardia* and
252 *Cryptosporidium* in backyard goats in Chandigarh have indicated that the majority of *Giardia* were
253 potentially zoonotic (Assemblages A and B), but *Cryptosporidium* infection were not identified
254 (Utaaker et al, unpublished results). These data suggest that in terms of zoonotic transmission of
255 *Cryptosporidium* and *Giardia* in Chandigarh with respect to livestock, goats may be of greatest
256 importance for *Giardia*, but bovids are probably of greatest importance for *Cryptosporidium*.

257 Veterinary urban hygiene in India has been pinpointed as an area of concern (Singh et al., 2013), and
258 our data here support this with respect to *Cryptosporidium* in cattle. Given the importance of
259 cryptosporidiosis in human health in India (e.g., Kotloff et al, 2013) and also that bovine health can
260 be seriously affected by this infection, this intestinal parasite should be of importance in One Health
261 studies in this region. However, as has been previously noted, in order to support effective One
262 Health research in India along with integrated zoonotic disease control, it is necessary that
263 substantial changes in behaviour, attitudes, and institutional policies are implemented (McKenzie et
264 al., 2016).

265 **5. Conclusion**

266 This study further emphasizes the status of *Cryptosporidium parvum* as a zoonotic pathogen, even in
267 India, where human cryptosporidiosis usually tends to be due to *C. hominis* infections. The
268 predominant *C. parvum* subgenotype that we identified in bovids has also been reported in people in
269 the same region. Although *Giardia* from bovids seemed to be less likely to be associated with
270 zoonotic transmission, the concomitant finding of Assemblage B in one calf indicates that
271 anthroponotic / zoonotic transmission between humans and bovids should not be excluded. We
272 speculate that the special cultural status of cattle in India may be partially responsible for increased
273 sharing of pathogens between cattle and humans, particularly in the urban environment.

274 **Conflict of interest statement**

275 No financial or personal relationship between the authors and other people or organizations have
276 inappropriately influenced this work.

277 **Ethics statement**

278 It is submitted that proper consideration has been given to any ethics issue raised.

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All authors: Kjersti Selstad Utaaker, Suman Chaudhary, Rajinder S. Bajwa, Lucy Robertson.

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VII

Not just a walk in the park: occurrence of intestinal parasites in dogs roaming recreational parks in Chandigarh, Northern India.

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Abstract

The population of stray dogs in India is on the rise. Chandigarh is a city in the North-west of India with numerous parks and recreational areas where stray dogs roam free. This allows for extensive human-dog interaction, which may pose a threat to human health.

The aim of this study is to determine the occurrence of gastrointestinal parasites of public health importance, and their seasonal variance, in canine faecal samples obtained from recreational parks in Chandigarh.

A total of 212 samples were collected from 10 parks in winter (January 2015; N=107) and during the monsoon season (September 2015; N=105), to assess the prevalence of intestinal zoonotic parasites and any seasonal variation in prevalence. The samples were analyzed for helminth eggs with McMaster counting chambers. Immunofluorescent antibody testing was used to analyze the samples for *Cryptosporidium* oocysts and *Giardia* cysts. The samples positive for *Giardia* were further genotyped by conventional PCR to determine their assemblage and zoonotic potential.

Among the 212 samples, strongyle-type eggs were found in 34 (16%), *Toxocara* eggs were found in 6 (2.8%), taeniid eggs in 1 (0.5%), *Cryptosporidium* oocysts in 4 (1.9%) and *Giardia* cysts in 51 (24%). The majority of the *Giardia* positive samples were canid specific assemblages. *Trichuris* eggs were not detected. The prevalence of *Giardia* cysts in fecal samples was significantly higher in winter, compared to the monsoon season, while the prevalence of helminth eggs did not alter with the time of sampling. There was no significant difference in the intensity of infection between the winter season and the monsoon season.

The study concludes that feces from dogs contaminating parks in Chandigarh do not usually contain parasite transmission stages that pose a significant risk to human health. However, the study emphasizes the importance of avoiding contamination with dog feces in public parks.

Key words: stray dogs, public health, zoonotic parasites, India

1. Introduction

Both stray dogs and intestinal parasites are found worldwide, and are known to be more prevalent in developing countries (Haque, 2007; Jackman and Rowan, 2007). Stray dogs have always been a part of the landscape of the developing world, but the exploding dog population has made it a global public health priority in recent times. There are about 700 million dogs worldwide (Hughes and Macdonald, 2013), and about 75 % of this population are defined as stray dogs, free to roam and reproduce (Massei and Miller, 2013). The population of stray dogs in India has been estimated to be around 25 million, and the number appears to be rising regardless of sterilization campaigns (WHO, 2016). Given the lack of veterinary control in the stray dog populations (e.g., vaccination and worm control) and their close contact with humans, these neglected animals may constitute an important reservoir of zoonotic pathogens (Traub et al., 2005).

Dogs may be hosts for various potentially zoonotic intestinal parasites, including *G. duodenalis, Toxocara canis, Toxascaris leonina, Ancylostoma caninum and Echinococcus* spp. Of the eight *G. duodenalis* genotypes (A-H) described to date, four have been found to infect dogs and other canids: Assemblage A and B, which have zoonotic potential, and C and D, which seem to be more specific for members of the canidae family. Although most studies with molecular data on *G. duodenalis* prevalence in dogs show that the canine specific genotypes are dominant (Beck et al 2012; Qi et al. 2016), there are exceptions that indicate that potentially zoonotic assemblages can predominate (Dado et al. 2012; García-Cervantes et al. 2016).

Among the zoonotic helminths infecting dogs, cestodes of *Echinococcus* spp. are of particular concern, due to the severity of infection in humans as aberrant intermediate hosts. Although the symptoms are usually less severe, important sequelae may occur when humans act as accidental hosts for the nematode, *T. canis*. A previous study from four sites in India found a low prevalence of both parasites, where they were absent in most locations, but with a 2.3 % prevalence of *Echinococcus* eggs were found in faeces from dogs from an arid mountainous region, and 3.2 % *Toxocara* eggs in dogs living in a humid temperate region (Traub et al., 2014).

An underreported and perhaps neglected zoonotic parasite in India is the hookworm *Ancylostoma caninum*, causing cuteanous larva migrans and eosinophilic enteritis in the aberrant human host. Although it is seldom reported from India, it may be endemic in some socioeconomically disadvantaged communities, where people cannot afford footwear and reside in areas heavily contaminated by faeces of stray dogs (Heukelbach et al., 2002) . Previous studies have shown a high prevalence of *A. caninum* in stray dogs in India, with prevalences ranging from 72 % to 99 % (Joshi

and Sabne, 1977; Malaki, 1966; Maplestone and Bhaduri, 1940; Sahai, 1969; Sahasrabudhe et al., 1969)

Currently, there are few reports with information about the prevalence of zoonotic intestinal parasites in stray dogs found in Northern-India, and given the rapid demographic changes in this region there is need for updated information about these important pathogens. This study therefore aimed to determine the occurrence of gastrointestinal parasites of public health importance, and their seasonal variation, in canine faecal samples obtained from recreational parks in Chandigarh, a city in Northern India.

2. Material and Methods

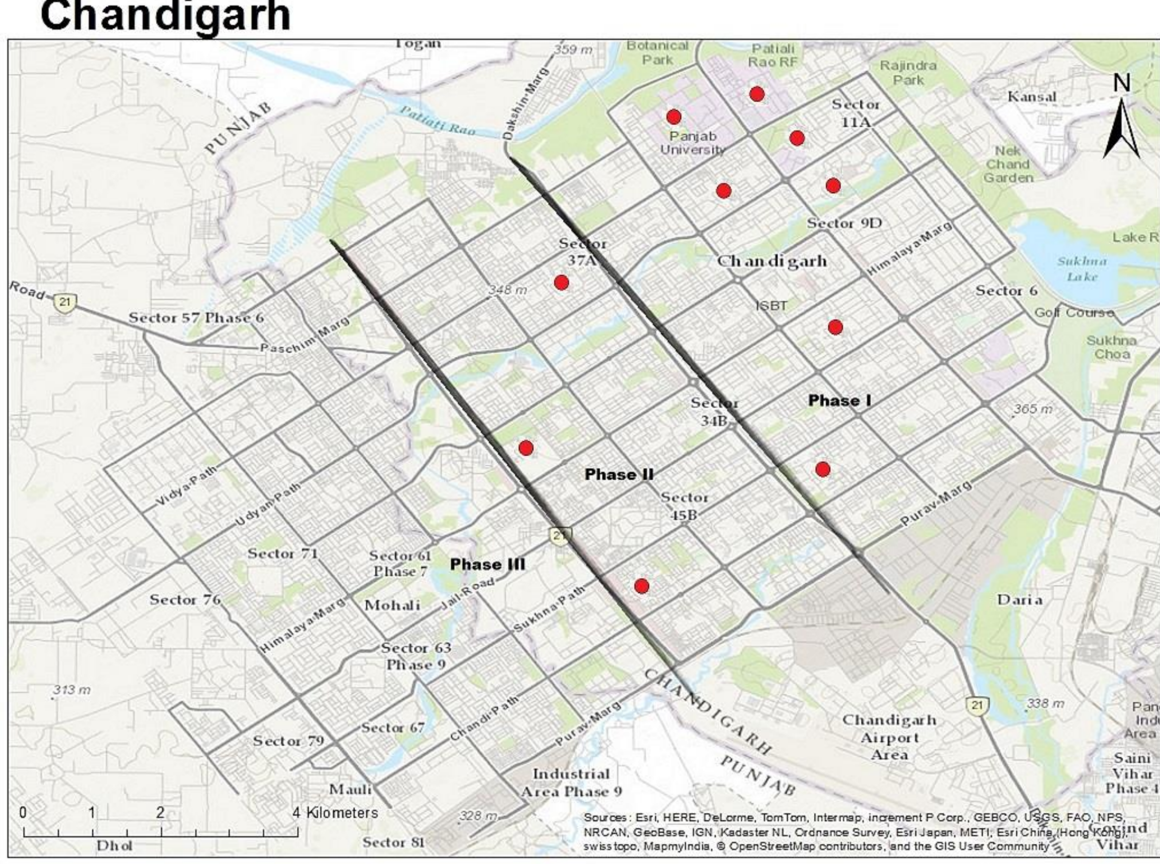
2.1 Sampling sites

Chandigarh is a city located north-west in India, and is a relatively new city, as its construction started in the early 1950s. It is known as the "city beautiful", as it is one of the greenest cities of India, with many parks and recreational areas. Chandigarh is also one of the fastest growing cities in India, and have a current population of over a million inhabitants (JNNURM, 2006). The city is divided into phases, and each phase has a different population density with phase I being the less populated with an increasing density throughout phase II and III; these phases thus also represent different socio-economic levels of the population, with the more crowded areas tending to have the poorest residents and the infrastructure being less well-developed.

Chandigarh has numerous public parks in every sector of the city, with an estimated total number of big and small parks close to 1900, and 35 % of the district Chandigarh defined as "green space" (Chaudhry et al., 2011), receiving many visitors on a daily basis. Currently, the city is facing problems with maintenance of these parks, and they often remain untended and littered (India, 2016).

Stray dogs often reside in these recreational parks, where they scavenge for food, sleep, reproduce, and defecate, and these parks are therefore important hot-spots for human-dog interactions. Samples were collected from 10 parks (see Figure 1) during the winter (January 2015; N=107) and the same parks were sampled again during the monsoon season (September 2015; N=105) to assess the prevalence of intestinal zoonotic parasites in the samples and any seasonal variation.

Figure 1: Map of sampling sites.



2.2 Sample collection

Faeces were collected from the ground of the parks where free roaming dogs had been observed. Samples were placed in plastic containers and transported directly to the laboratory where they were re-suspended in 5 % potassium dichromate and kept at 4°C before analysis.

2.3 Sample preparation

Before analysis, potassium dichromate was removed by washing and centrifugation steps and a sub-sample of 3 grams was rinsed with phosphate buffered saline (PBS). The pellet was then homogenized with 57 mL water and sieved using metal sieve with a pore diameter of 425 µm. The suspension was divded in two 10 ml tubes with screw cap and centrifuged at 1550 rfg for 3 minutes. The supernatant was discarded and the pelleted samples were stored at 4°C until further processing.

2.4 Parasitological analysis

2.4.1 McMasters for analysis for helminth eggs

The concentrated pellet from one of the 10 ml tubes was resuspended in NaCl/$ZnCl_2$ solution. McMaster counting chambers (supplier etc) were filled with this suspension and read at 40x and 100x magnification for detection of parasite eggs.

2.4.2 Immunofluorescent antibody testing (IFAT) for analysis for *Cryptosporidium* oocysts and *Giardia* cysts

Between 5-20 µl of homogenized and sieved faecal material from the other 10 ml tube was placed on a microscope slide using plastic bacteriological loops. Samples were left to dry before fixation with methanol and staining with 15 µl of monoclonal antibodies labelled with FITC against *Cryptosporidium* oocysts and *Giardia* cysts (AquaGlo, Waterborne Inc. USA). After 30 minutes of incubation at 37°C in a humid chamber, the slides were carefully washed with distilled water to remove excess antibodies, then covered with a cover slip and screened under a fluorescent microscope with the following filter settings: FITC: emission- 490, excitation – 525.

The samples were graded after counting the number of cysts/oocysts per field of view at x20 magnification according to the system in table 1.

Table 1: Grading of cyst / oocyst count immunofluorescent microscopy

Cyst / oocyst count	Grading
1-9	+
10 – 50	++
>51	+++
>100	++++

2.5 Molecular methods for Assemblage identification of *Giardia* cysts

DNA was isolated from *Giardia* positive samples. From the samples collected during the winter season, only the slides containing the cysts were available for DNA extraction. These slides were prepared as according to Robertson et al (2009), Briefly, the coverslip from each slide was carefully removed and retained, whilst 25 µl aliquots of AL lysis buffer (Qiagen GmbH, Germany) were added to the slide , which were then scraped off using a sterile scalpel blade. The buffer and scrapings were pipetted into a microcentrifuge tube. This process was repeated four times, and then the coverslip

was replaced onto the slide and re-screened. For each slide, no cysts could be detected after scraping.

The contents of each microcentrifuge tube containing slide scrapings were mixed with 150 µl Tris-EDTA buffer (100 mM Tris and 100 mM EDTA) and incubated at 90°C for one hour, before the DNA was isolated using QIAamp DNA mini kit (Qiagen GmbH). Apart from an overnight step at 56°C with proteinase K, the procedure followed the manufacturer's instructions.

For the samples collected during the monsoon season and found positive by faecal smear, *Giardia* cysts were isolated using an in-house immunomagnetic separation method (IMS) using Dynabeads® (GC-Combo, Life Technologies, Carlsbad, CA) as published by Robertson et al., (2006), and Utaaker et al, (2015); 10 µl anti-*Giardia* beads, 10 µl anti-*Cryptosporidium* beads, 80 µl Sur-Modics StabilZyme® , 20 µl SL Buffer B and 100µl Buffer Q4 were used to generate 55 µl of purified sample from approximately 200 mg of the faecal pellet. Each microcentrifuge tube containing *Giardia* cysts was re-suspended in 150 µl Tris-EDTA buffer and held at 90°C for one hour, before the DNA was isolated as described above.

DNA was finally eluted in 30 µl of PCR grade water and stored at 4 °C.

2.5.1 Multi-locus genotyping of *Giardia* isolates

Several genes were used to investigate the *Giardia* positive samples by conventional PCR, but only two primer sets showed a positive result: the glutamate dehydrogenase (*GDH*) gene as described by Read et al. (2002) and the small subunit ribosomal RNA (*SSU RNA)* according to Hopkins et al. (1997).

The other primer sets for the triosephosphate isomerase (TPI and TPI-D) and β-giardin (BG) genes did not yield a positive result with the protocols of Sulaiman et al, (2003) Lebbad et al (2010) and Lalle et al (2005) .

For all genes, the following PCR mixture was used: 1 µL of each primer (10 pmol), 0.2 µl of bovine serum albumin (20 mg/ml), 8.3 µl of water, 12.5 µl of HotStartTaqmaster (QIAGEN® GmbH, Germany), and 2 µl of template, giving a final reaction volume of 25 µl. For each set of reactions, a negative control (water) and a positive control (DNA from *G. duodenalis* H3 isolate belonging to assemblage B, Waterborne Inc., New Orleans, USA) were included.

PCR products were run on 2 % agarose gels and stained with SYBRsafe® DNA gel stain under UV radiation. Positive samples were purified using ROCHE® high pure PCR product purification kit, and puri-

fied products were for sequencing on both strands by a commercial company (GATC biotech, Germany). Sequences were assembled and manually examined using Geneious 10.1.2 software and sequence comparisons conducted using NCBI BLAST.

3. Statistics

A database of results was created in Excel. Prevalence rates and associations with sampling occasion (seasonality) were compared by contingency table analysis (Chi square). Mann-Whitney U-tests were used to compare the intensity of infection with strongyle-type eggs according to sampling occasion, whereas for *Giardia* cysts, shedding intensity was categorised (see Table 1) and associations by sampling occasion investigated by contingency table analysis.

4. Results

4.1 Prevalence of different parasites

Among the total of 212 samples, strongyle-type eggs were found in 34 (16 %), *Toxocara* eggs were found in 6 (2.8 %), taeniid eggs in 1 (0.5 %), and *Giardia* cysts in 51 (24 %). An overview by park location and season is provided in Table 2. The strongyle-type eggs are assumed to be *Ancylostoma caninum*, the dog hookworm, but this was not verified by further testing. *Cryptosporidium* oocysts (1.9%) was found in low amounts in the samples collected during the winter season, and *Trichuris* eggs were not detected in any of the samples.

The prevalence of helminth eggs (*Toxocara* and strongyle-type eggs) found in the samples did not alter with time of sampling (winter or monsoon). However, the prevalence of *Giardia* cysts in faecal samples was significantly higher during the winter season (28.7 %) than during the monsoon season (16.9 %).

Table 2: Helminth eggs and *Giardia* cysts detected in faeces from parks, by park location and sampling occasion

Location (park ID)	**No. samples analysed (winter, monsoon, and total)**	**Winter sampling (no. samples positive with different helminths and/or *Giardia* cysts)**	**Monsoon sampling (no. samples positive with different helminths and/or *Giardia* cysts)**
Sector 10	Winter= 10 Monsoon= 10 Total = 20	Strongyle-type eggs (n=3); *Giardia* cysts (n=1) *Toxocara* eggs (n=1)	*Giardia* cysts (n=1)
Sector 11	Winter=10 Monsoon=10 Total =20	*Giardia* cysts (n=2)	Strongyle-type eggs (n=2); *Giardia* cysts (n=1)
Sector 12	Winter=10 Monsoon=10 Total =20	Strongyle-type eggs (n=1)	Strongyle-type eggs (n=5); *Toxocara* eggs (n=3) *Giardia* cysts (n=4)
Sector 14	Winter=10 Monsoon=11 Total =21	Strongyle-type eggs (n=1);	*Giardia* cysts (n=1)
Sector 15	Winter= 10 Monsoon=11 Total = 21		Taeniid eggs (n=1) *Giardia* cysts (n=6)
Sector 18	Winter= 10 Monsoon=10 Total =20	Strongyle-type eggs (n=4); *Giardia* cysts (n=6)	
Sector 30	Winter= 10 Monsoon= 14 Total = 24	Strongyle-type eggs (n=1); *Giardia* cysts (n=3)	
Total Phase I	**Winter=70** **Monsoon=76** **Total= 146**	**Strongyle-type eggs (n=10);** ***Giardia* cysts (n=12)** ***Toxocara* eggs (n=1)**	**Strongyle-type eggs (n=7);** ***Giardia* cysts (n=13)** ***Toxocara* eggs (n=3)**
Sector 37	Winter= 15 Monsoon=10 Total =25	Strongyle-type eggs (n=6); *Toxocara* eggs (n=2) *Giardia* cysts (n=14)	*Giardia* cysts (n=3)
Sector 43	Winter=10 Monsoon=10 Total =20	*Cryptosporidium* oocysts (n=4)	Strongyle-type eggs (n=7); *Giardia* cysts (n=2)
Sector 46	Winter=12 Monsoon=9 Total =21	Strongyle-type eggs (n=4); *Giardia* cysts (n=3)	Strongyle-type eggs (n=1); *Giardia* cysts (n=4)
Total Phase II	**Winter=37** **Monsoon=29** **Total =66**	**Strongyle-type eggs (n=10);** ***Giardia* cysts (n=17)** ***Toxocara* eggs (n=2)**	**Strongyle-type eggs (n=8);** ***Giardia* cysts (n=9)** **Taeniid eggs (n=1)**
TOTAL	**Winter=107** **Monsoon=105** **Total =212**	**Strongyle-type eggs (n=20;** ***Toxocara* eggs (n=3)** **Taeniid eggs (n=0)** ***Giardia* cysts (n=29)**	**Strongyle-type eggs (n=15);** ***Toxocara* eggs (n=3)** **Taeniid eggs (n=1)** ***Giardia* cysts (n=22)**

4.2 Intensity of infection

The number of eggs per gram detected in positive samples for helminths and number of cysts per field of view for *Giardia* cysts was used as a reflection of intensity of infection.

	Winter season	Monsoon season
Strongyle eggs	10-150	10-1,260
Toxocara	10-250	10-10,000
Taenia	880	
Giardia	>G++	<G+

The concentration of taeniid eggs in the single sample where these eggs were detected was 880 eggs per gramme (epg). For *Toxocara* eggs, the concentration of eggs in the samples was from 10 epg, up to 150 epg in the winter season samples, and up to 1260 epg in the monsoon season samples.

For the strongyle-type eggs, the concentration of eggs ranged from 10 to 250 epg (mean: 70 epg; median 45 epg) in the samples taken during the first sampling (winter season), and from 10-10000 epg (mean: 966 epg; median 100 epg) at the second sampling (monsoon). Mann-Whitney U-tests revealed no significant difference between the medians (p_1=0.778, p_2=0.1556)

For *Giardia* cyst excretion rate, of the 25 positive samples identified during the first sampling (winter season), the majority (15) were G++ or above (5 with G++ and 10 with G+++), with only 10 G+. In the second sampling (monsoon season), the opposite picture emerged with the majority of the 18 positive samples being G+ (n=12), and only two G++ and four G+++. However, no significant associations between intensity of cyst shedding and sampling occasion was detected.

The overall prevalence from both sampling occasions differed between the two phases where samples were collected. From phase I, strongyle eggs had a prevalence of 12 %, *Giardia* cysts werefound in 17 %, and *Toxocara* eggs were found in 3 % in the samples. For phase II, these numbers were 27 % (strongyle eggs) 39 % (*Giardia* cysts) and 3 % (*Toxocara* eggs). When compared with a chi-square test, the difference in prevalence of strongyle eggs and *Giardia* cysts were statistically significant between the two phases.

4.3 Molecular analyses

PCR analysis was attempted on 44 *Giardia* samples, 27 from the winter samples and 17 from the monsoon samples; positive results were obtained from 19 samples (43 %) at the SSU gene and from 1 sample at the GDH gene.

Of the sequences obtained, the majority were canid specific assemblages (13 Assemblage C, 4 Assemblage D, 2 Assemblage A, 1 Assemblage B, and 1 Assemblage A). The sequences have been deposited in GenBank under the Accession numbers: MF281098, MF153912, MF153911, MF281090, MF153913, MF153910, MF281091, MF153914, MF281092, MF281094, MF281096, MF281089, MF281093, MF153916, MF281097, MF153909, MF281095 and MF153397.

Of the sequencing results obtained from the winter samples, all were canid specific assemblages (7 Assemblage C and 1 Assemblage D).

5. Discussion:

The results of this study demonstrated a moderate prevalence of *Giardia* and strongyle-type eggs in dog faeces obtained from public parks in Chandigarh, and a low prevalence of *Toxocara* eggs.

Giardia cysts were found in 24 % of the faecal samples, though the actual prevalence may be an underestimate as *Giardia* cysts are excreted intermittently.

Although the relevance of dogs as a zoonotic source of human *Giardia* is generally considered low (Tysnes et al., 2014), the majority of studies have been conducted in Europe, North America, and Australasia, and there are relatively few studies from less developed countries, including India, that investigate the prevalence and zoonotic potential of *Giardia* in dogs in this country. A study from a tea-growing community in North-East India found that subassemblage AII was the dominant genotype among humans and dogs. However, over 30 % of the dogs examined also had *Ascaris lumbricoides* eggs of high numbers in their faeces, suggesting that the dogs a may as well be mechanical disseminator of parasites through coprophagy (Traub et al., 2004; Traub et al., 2003). Some studies have found that genotypes are shared between humans and dogs (Inpankaew et al., 2007; Traub et al., 2009), while others have not (Cooper et al., 2010; Lebbad et al., 2008). It seems that the transmission of *Giardia* between dogs and humans and the occurrence of zoonotic Assemblages in dogs is determined by factors specific to each endemic area, and that these vary greatly.

In our study, the majority of the *Giardia* were apparently canid-specific and not associated with zoonotic transmission. However, only a limited number of samples gave positive PCR results and were successfully sequenced. This seems to be a common challenge in molecular studies of canine giardiasis (Leonhard et al., 2007; Sommer et al., 2015), which warrants further investigations to improve the molecular tools used on *Giardia* isolates from dogs. Although previous reports have suggested that dogs may pose as a reservoir for *Giardia* infections in Asia, this seems not to be the case generally in Chandigarh.

Strongyle-type eggs

The strongyle-type eggs detected in our study were assumed to be *Ancylostoma caninum*, dog hookworm. Traub et al (2014) found varying prevalences across India in a study on stray dogs, ranging from 4.7% to 70.2%. Factors influencing distribution of hookworms are likely to be climatic, and dry winters may be detrimental to survival of *Ancylostoma* larvae in the environment. However, *A. caninum* can undergo hypobiosis within the host tissue and thus evade unfavourable climatic conditions and reactivate once environmental conditions are more suitable for its survival, giving this species a significant competitive advantage over other hookworm species. In addition to being pathogenic in dogs, canine hookworms may also produce a temporary pruritic popular skin rash known as cutaneous larva migrans (Maplestone, 1933). In addition, as hookworms previously found in North Indian dogs have ultimately been identified as *A. ceylanicum* (Traub et al., 2007), the hazards may be more detrimental to humans than a rash. In addition to severe anaemia, *A. ceylanicum* may cause, impaired physical and cognitive development of children. Children may also be at greater risk to aquire such infection as they play on the ground in these parks alongside the roaming dogs. Further molecular studies are necessary to confirm if dogs may act as a reservoir for *A. ceylanicum* in Chandigarh.

Toxocara

The prevalence of *Toxocara* eggs in this study was lower than expected, as the global prevalence of this common nematode in dogs is relatively high. A dog infected with adult worms of *T. canis* may shed thousands of eggs each day with faeces, and a high prevalence would be expected in soil of urban areas where there is a relatively large number of dogs with access to limited green space for defecation (Overgaauw, 1997); studies conducted in parkland of cities worldwide have, in many cases, found considerable soil contamination with eggs of *Toxocara* spp (Genchi and Traldi, 1994; Kleine et al., 2017; Otero et al., 2017).

Studies from Brazil, Italy, and Spain have shown *Toxocara* prevalences in dogs ranging from 8.7 % to 17.7 % (Katagiri & Oliveira-Sequeira, 2008; Martinez-Moreno et al., 2007; Zanzani et al., 2014). In India, a study from Uttar Pradesh reported a *Toxocara* prevalence of 24.3% of dogs in this area, and both stray and pet dogs were examined (Sahu et al., 2014). In contrast, a study from four different climatic locations in India found prevalences ranging from 0 to 3.2 % (Traub et al., 2014). As the areas chosen for sampling in our study are recreational parks, rather than streets or wasteland, the low prevalence could reflect that the samples collected have been from pet dogs, whose owners may have been advised to deworm their pets on a regular basis. This may partly explain the low prevalence, although the results seem to be in concordance with Traub et al (2014). In addition, as the samples were picked up after defecation and the dogs were not observed, the smaller faeces from puppies may have been

overlooked and not sampled at all. As adult dogs tend not to have adult worms in the intestine, the results may show the *Toxocara* prevalence in mainly adult dogs.

The apparent absence of *Trichuris vulpis* eggs in our study supports the theory of Traub et al (2002), who suggested the absence of this parasite in Indian dogs. This absence remains unexplained as other host–specific species within the genus *Trichuris* occur endemically throughout the country in humans and livestock.

We also found some samples with a few *Cryptosporidium* oocysts, and, as with the *Toxocara*, the low prevalence may reflect that the samples were mostly derived from adult dogs, which are less likely to have active *Cryptosporidium* infections. Due to the small number of oocysts, and only slides being available for DNA isolation, molecular methods were not applied on these samples. The low prevalence of *Cryptosporidium* in these samples seems to correlate with that of Traub et al (2002), who found a 2.5% prevalence of *Cryptosporidium* in dogs in a tea estate in Assam, India, though Daniels et al (2015) found a 17% prevalence of *Cryptosporidium* in dogs in Odisha, India. Molecular methods were either not applied or successful in these studies, leaving the zoonotic potential of *Cryptosporidium* shed by dogs in India yet undefined.

Whether the faeces were from pet dogs or stray dogs cannot be ascertained. Although the probability that the faeces collected were from canines is high is supported by the results of the *Giardia* genotyping analyses, it is not impossible that some of the faeces may have been from humans or other animals.

6. Conclusion

Our results suggest that faecal samples from dogs contaminating parks in Chandigarh do not usually contain parasite transmission stages that pose a significant risk to human health. Further work that focuses on stray dogs in particular and determines the actual species of Strongyle type eggs is recommended to clarify their position. In addition, it is recommended that parks in Chandigarh are cleaned regularly, that the stray dog population is controlled, and that dog owners are strongly encouraged to take responsibility for clearing up after their dogs.

Conflict of interest statement

No financial or personal relationship between the authors and other people or organizations have inappropriately influenced this work.

Ethics statement

It is submitted that proper consideration has been given to any ethics issue raised.

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Additional articles

VIII: Utaaker, K. S. & Robertson, L. J. (2015). Climate change and foodborne transmission of parasites: A consideration of possible interactions and impacts for selected parasites. *Food Res. Int.*, 68: 16-23.

IX: Robertson, L. J., Utaaker, K. S., Goyal, K. & Sehgal, R. (2014). Keeping Parasitology under the One Health umbrella. *Trends Parasitol.*, 30 (8): 369-372.