Full GMM Risk Assessment 1424

FullSerialNo	GMM1424
Version	1
Title	Work with Cryptosporidium parum
Final_class	Class 2
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Building	Wellcome Trust Biocentre
Lab_No	1L2-12
Name of assessor	
Approval_date	25/08/2017
Review date	25/08/2018

1. Brief description of project

Diarrhoeal disease is a major cause of mortality, morbidity, and developmental stunting in young children worldwide. The Global Enteric Multicenter Study (GEMS) recently revealed an immense and previously under-appreciated disease burden resulting from infection with the parasite Cryptosporidium. Of the pathogens that cause severe diarrhoeal disease in children less than two years of age, Cryptosporidium is second only to Rotavirus. Unlike Rotavirus however, there is no vaccine for cryptosporidiosis. Infection with Cryptosporidium as a child has life-long impacts. Chronic cryptosporidiosis contributes to malnutrition and growth stunting. Poor nutrition during the first two years of life lowers vaccine efficacy and impairs cognitive development. Cryptosporidiosis can also cause chronic, life-threatening diarrhoea in immunocompromised adults, ie AIDS patients. For young children and immunocompromised adults there are no efficacious drugs.
Cryptosporidium is an ubiquitous water borne pathogen that is contracted upon ingestion of contaminated food or water. Unlike other pathogens, Cryptosporidium is resistant to almost all disinfectants including in the case of water treatment, chlorination. Recreational water facilities that use chlorination for sanitation are often the source of Cryptosporidium outbreaks in the developed world. Two species of Cryptosporidium infect people: C. hominis and C. parvum. While C. hominis is limited to humans, C. parvum also infects cows, sheep, and goats. C. parvum is a significant source o illness in young calves in the UK. There is also no efficacious drug for cattle cryptosporidiosis. There is a great need to develop drugs, vaccines, and cost-effective water treatments for Cryptosporidium. The objectives of this work are to use genetic tools to study the basic biology/biochemistry of the parasite, and to identify and validate potential drug candidates for cryptosporidiosis.
There are significant challenges in working with Cryptosporidium. This pathogen cannot be continuously cultured in the lab; therefore, we depend on animal infections to propagate and produce the organism. Also, until recently there were no molecular biology tools available to study the genetics of this pathogen. Hence, our understanding of the basic biology and biochemistry of this parasite is sparse. Generating genetically modified Cryptosporidium allows us to determine the function of genes and understand how they are related to disease. We have developed genetic tools that allow us to

modify wild type Cryptosporidium, for example by deleting or tagging genes, and to produce transgenic parasites. We plan to use these tools to make transgenic organisms to study genes related to the life cycle of Cryptosporidium and to identify and validate potential drug targets.
The aims of this work include 1A) identifying genes expressed in various life cycle stages, especially transmission, 1B) identifying genes that are the target of anti-cryptosporidial compounds, 2) generating transgenic organisms for these candidate genes and 3) describing the function of these genes in Cryptosporidium biology, transmission, and drug susceptibility.

The life cycle we are most interested in studying is the “oocyst”. Cryptosporidium is transmitted via a fecal-oral route and is shed in fecal material as an oocyst. The oocyst wall renders the parasites resistant to most environmental stressors, including virtually all disinfectants (ethanol, detergent, bleach i.e. chlorination). Currently very little is understood of how the oocyst wall is formed and why it is so resistant to disinfection. Understanding how the oocyst is formed and describing the role of various genes in the transmission process will reveal new interventions for water treatment and drug therapies.
Using genetics, we have also generated “reporter” strains that express reporter genes (GFP, mCherry, luciferase, etc) that aid in visualizing and quantifying Cryptosporidium. These are especially useful in the context of testing potential anti-cryptosporidial compounds. There is currently only a single drug approved for use against cryptosporidiosis, however it provides no relief for young childrer or immunocompromised adults. Therefore, there is a great need for anti-cryptosporidial drug discovery. Use of reporter strains will greatly enhance the ability to screen compounds both in vitro and in vivo. Once anti-cryptosporidial compounds are identified, genetic tools will be used to validate drug targets and understand the mode of action of the compounds.
Currently there is no continuous in vitro culture system for Cryptosporidium. Therefore, to propagate Cryptosporidium we depend on animal infections. Transgenic parasites are maintained in mouse animal infections in the lab. This provides a steady supply of organisms with which to study basic biology and biochemistry, and for drug discovery. Cryptosporidiosis is a threat to both developing and developed nations, and lack of preventative methods and interventions underscore the need to better understand this important pathogen.
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2a. Hazards to human health associated with the recipient microorganism

Cryptosporidium is an intracellular pathogen whose growth and replication is entirely dependent on its host. There are 32 identified species of Cryptosporidium however two species, C. parvum and C. hominis, cause disease in humans. There is no continuous in vitro culture system for any species of Cryptosporidium, however short-term in vitro culture systems exist for C. parvum and C. hominis. We co-culture C. parvum transgenic organisms with HCT-8 cells (human ileocecal adenocarcinoma cell line). Other validated in vitro culture cells lines and systems may be used if they become available (including but not limited to Caco-2 cells, hollow-fiber-culture systems, ‘gut-on-a-chip’, and mouse/human intestinal organoid cultures). In all instances, intracellular stages of the pathogen are easily decontaminated with common disinfectants (ethanol, detergent, etc).

We use C. parvum, rather than other species that don’t infect humans because C. parvum is the species amenable to genetic manipulation and easily maintained in the lab. Additionally the Iowa II strain of C. parvum is the only species and strain regularly available from industry/academic sources. Because there is no continuous in vitro culture system, we depend on industry/commercial sources to produce large numbers of Wild Type organisms from infected cows. We then use these Wild Type organisms as a starting point to generate transgenic parasites. Transgenic parasites (insertion or deletion mutants) are initially selected with paromomycin in infected animals, and are maintained in the presence of paromomycin to select for only growth of transgenic parasites. In this way, reversion to Wild Type is highly unlikely. Finally, we do not have enough knowledge of the life cycle, virulence factors, and immune response to Cryptosporidium to engineer a disabled or attenuated Iowa II strain. We will utilize an attenuated strain if one becomes available.

Although Cryptosporidium parvum is a human pathogen, if handled properly, poses little risk to human health. The form of the parasite transmitted between hosts, the oocyst, is resistant to most chemical disinfectants (ethanol, detergent, bleach) but is neutralized with 3-6% hydrogen peroxide, UV, autoclaving, or simply air-drying. Oocysts will be stored in a specific, easily identified location in the lab and will be stored in two layers of containment (with internal absorbent material) to avoid spilling. Once oocysts are co-cultured with HCT-8 cells, common disinfectants are sufficient to decontaminate cultures.

To propagate transgenic Cryptosporidium we depend on animal infections (cow, sheep, mice); in the lab we use use immunocompromised mice for this purpose. While these mice are themselves transgenic animals, they are a standard genotype widely commercially available. Infection with Cryptosporidium does not alter the genome of the mice and use of these animals pose no inherent risk to human health. If an in vitro continuous culture model becomes available, we will use this technology to replace some of the animal infections (in vitro culture systems are more easily contained and decontaminated).

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2b. Hazards to human health arising directly from the inserted genetic material

Both Wild Type and transgenic Cryptosporidium parvum will be used in the lab (wet lab and animal facility). Transgenes expressed by Cryptosporidium parvum are not known to be harmful, and are not transferred from Cryptosporidium to its host (human cells in co-culture or infected animal—humans included). A list of common transgenes is below and includes reporter genes regularly used in biological research:

- Fluorescent reporters: YFP, GFP, mCherry, mNeon, RFP, Beta-galactosidase
- Luciferase reporters: NanoLuciferase, Red-Shifted Firefly Luciferase, Gaussia Luciferase
- Drug Resistance Markers: Neomycin Resistance (confers resistance to paromomycin; paromomycin is an effective drug for treatment of cryptosporidiosis in immunocompromised mice, but not in human cryptosporidiosis)
- Promoters/Elements that regulate gene expression: C. parvum enolase promoter and 3' untranslated region, TIR1 (O. sativa), Auxin-binding domain (O. sativa), Cre Recombinase (Bacteriophage), Cas9 (S. pyogenes), C. parvum U6 promoter, FKBP and destabilization domain, CMV promoter, BirA (biotin ligase), APEX (ascorbate peroxidase)
- Epitopes: HA, V5, GST, c-Myc, Ty

HCT-8 cells may be transfected with transgenes listed above to generate reporter host cells to be used in co-cultures with Cryptosporidium.

2c. Hazards to human health arising indirectly from the inserted genetic material

None that are known

2d. Hazards to human health arising from transfer of genetic material to a related

We use the inherent homologous recombination machinery of C. parvum to integrate transgenes at specific locations in the genome. All transgenes used to generate transgenic Cryptosporidium strains are chromosome borne. Currently there is only a single drug resistance marker for Cryptosporidium, therefore only a single cassette of transgenes may be introduced into a strain. Transgenic C. parvum are maintained under drug pressure, selecting for growth of only transgenic organisms. Additionally, all transgenic strains are produced from the same parental C. parvum Iowa II strain, so recombination or gene transfer from transgenics to Wild Type does not increase the genetic diversity. Cryptosporidium do not stably maintain plasmids, therefore transgenes are all chromosome borne.

Cryptosporidium are water borne, but cannot replicate in the environment. Cryptosporidium parvum can only survive and grow inside the host organism (cow, sheep, humans, immunocompromised

mice).

3. Assign a provisional containment level

Containment Level 2

4a. Hazards to the environment associated with the recipient microorganism

Cryptosporidium is a standard CL2 pathogen known to infect a range or organisms, including humans (please see section 2a above).

4b. Hazards to the environment arising from the genetic material

All the transgenes to be introduced are non-harmful, but as per section 2b above, drug resistance cassettes will be used. Neomycin resistance does confer resistance to paromomycin, which is an effective drug in the treatment of infections in immunocompromised laboratory mice, but is not effective in human infections.

5a. Brief description of nature of work (include maximum culture volumes)

Using newly available molecular tools for transfection and genetic engineering, it is now possible to interrogate specific parasite genes by knockout or modification to unravel their role in Cryptosporidium biology. The aims of this work include 1A) identifying genes expressed in various life cycle stages, especially transmission, 1B) identifying genes that are the target of anti-cryptosporidial compounds, 2) generating transgenic organisms for these candidate genes and 3) describing the function of these genes in Cryptosporidium biology, transmission, and drug susceptibility.

To generate a stable transgenic parasite, reporter and selection constructs are introduced into sporozoites by electroporation followed by infection of highly susceptible mice. Because transfected sporozoites are no longer encased in the protective oocyst wall, their ability to survive the acidic environment of the stomach is reduced if infected orally. Therefore, we surgically inject transfected sporozoites directly at the site of infection—the small intestine. Surgery is only required when transfected sporozoites are initially used to infect mice. Stable maintenance of transgenic parasites is selected for by growth in the presence of paromomycin, which is administered to mice in the drinking water.

Following infection, oocysts are shed with the feces and transgenic oocysts can be appreciated and quantified by measuring Nluc activity directly in the fecal material collected. Similarly, successful transfection is evident by Nluc activity from tissue culture. In a typical mouse infection, transgenic organisms emerge over the course of two to four weeks and oocysts can be isolated from fecal material for downstream experiments in tissue culture (PCR, Western Blots, mass spec analysis, immunofluorescence, etc) or animals. After transgenic oocysts have been recovered, subsequent mice can be orally infected by simple gavage with oocysts—similarly transgenic oocysts can be used to infect tissue culture for in vitro experiments. Oocysts are treated with bleach after isolation and before use in downstream experiments.

Typically, 1.5 ml of fecal material is collected from an entire cage of mice every three days for a

month at a time, producing 10-15 ml of infected fecal material per cage of mice per month. Oocysts are routinely isolated from fecal material and used within three months of production. This ensures manageable amounts of oocysts are stored at any one time (estimated <500 ml infected fecal material, never stored in aliquots of more than 50 ml). Additionally, purified wild type oocysts are also stored in the lab at <5x109 oocysts total at any one time. All fecal material and purified organisms are stored at 4C as described in 5g. Wild type and transgenic oocysts will be stored in the fecal processing room, to which access is limited.

Once oocysts are co-cultured with human cells and the parasite is intracellular, the pathogen is easily disinfected. This means that culture production for use in large scale screen does not pose additional safety risks. Cryptosporidium is easily contained and cannot be transmitted in aerosols because it is inactivated by drying (please see section 2a).

5b. Is a microbiological safety cabinet or isolator required to protect the worker from aerosol transmission?

No

5c. Waste disposal

Water bottles and used feed must be autoclaved prior to washing or disposal by placing in taped closed - autoclavable biohazard bag. Autoclave using SLS standard conditions.
Dirty bedding and isolator units must be autoclaved prior to disposal by placing in taped closed, autoclavable biohazard bag - autoclave using SLS standard conditions.
Flasks, pipettes, plates, glassware, spent media, etc will be autoclaved prior to washing or disposal by placing in taped closed, autoclavable biohazard bag - autoclave using SLS standard conditions.
Spills/waste (media, feces, tissue, towels, etc) from sample collection will be placed into a biohazard bag in a biohazard bin; other surface spills will be treated with 3-6% H2O2 application for 20 minutes.
Any needles/blades used for work or unintentional sharps (from broken glass/plastics) will be placed in a sharps container which is then disposed of via standard SLS clinical sharps disposal route.
Biosafety cabinet/work areas - 3-6% H2O2 application for 20 minutes, or use of Diversey Oxivir Disinfectant Spray (or similar) and wipe down following handling of parasites on surfaces or nonporous materials contaminated with parasites. H2O2 made fresh every 30 days.
Biosafety cabinet/work areas - 3-6% H2O2 application for 20 minutes and wipe down following handling of parasites on surfaces or nonporous materials contaminated with parasites. H2O2 made fresh every 30 days.

5d. Are sharps required? Yes or no. If yes, justify use.

Yes, when a new transgenic strain is produced, naïve mice are surgically infected with genetically modified Cryptosporidium. Surgical infection requires opening of the peritoneal cavity, injection of Cryptosporidium into the small intestine, and closure of the incision site with sutures. Because Cryptosporidium are not blood-borne pathogens, but transmitted via fecal-oral routes, infection with Cryptosporidium cannot occur as a result of use of sharps during surgical procedures. Surgical procedures are required for only the initial infection; gavage is used for subsequent infections.

5e. If the work involves experimental infection of animals is it known if the animal will

shed the GM microorganisms?
If yes, give details and measures to prevent exposure.

Yes, C. parvum has a fecal-oral transmission cycle and infected mice will shed oocysts in their feces. Mice will be housed in isolation cages with filtered ventilation, isolated food and water sources, and will only be handled in a biological safety hood by staff wearing proper PPE over scrubs (gloves, gown, face mask, shoe covers). Cages, food, water, and housing material will be handled with gloves and will be autoclaved directly after use. PPE will be removed and autoclaved upon exit from animal room. Hands will be thoroughly washed twice before exiting the animal facility: once in the animal room and again after exiting the animal room. Work surfaces (biological safety cabinet, procedure areas) will be decontaminated with 3-6% hydrogen peroxide for 30 minutes and allowed to air dry (UV will also be used if available). Staff will not eat or drink in the animal facility and should use PPE appropriately to minimize skin exposure.

5f. If the work involves experimental infection of plants what is known about the likely route of transmission of the GM microorganisms?

Plants cannot be infected with Cryptosporidium.

5g. Where will the GM microorgansim be stored?

In the lab, a specific room (with a door) will be designated for processing of infectious fecal material (proposed space MSI L2-56). A sticky floor mat will be used to prevent trafficking material outside of the fecal processing room. Additionally the fecal processing room will have a dedicated set of lab coats and goggles to be worn only in that space. Gloves will be changed frequently and staff will wash hands at the wash hand basin inside the fecal processing room after removing PPE but before exiting

Cryptosporidium oocysts are shed from infected animals in the feces. Cryptosporidium are killed wher stored at freezing temperatures, and become inactive if stored at room temperature. Cryptosporidium oocysts are stable at 4C and will be stored either as purified oocysts (in PBC or 2.5% potassium dichromate) or as infected fecal material. Both wild type and genetically modified Cryptospoirdium will be stored in a dedicated refrigerator in the fecal processing room. This refrigerator will be clearly labeled as containing both wild type and GMO Cryptosporidium. Additionally, Cryptosporidium samples are stored individually in smaller containers (1.5 ml or 50 ml centrifugation tube, depending on sample size) along with an absorbent material within a larger hard plastic container (clear plastic box) to prevent spills.

5h. How will the GM microorganism be transported within/between buildings to minimise risk of spillage/escape?

Cryptosporidium will be transported similarly to storage methods. Cryptosporidium samples are aliquoted in smaller containers (1.5 ml or 50 ml centrifugation tube, depending on sample size) along with an absorbent material within a larger hard plastic container (clear plastic box) to prevent spills. The larger plastic container may contain cool packs to maintain the temperature at 4C. Cool packs are preferred over ice as they are easily decontaminated.

5i. Will staff/students receive any vaccination or health surveillance? If yes, give details.

There is no vaccine for Cryptosporidium prevention. Infection causes acute gastroenteritis. Symptoms include diarrhea without red blood cells, abdominal pain, cramps, fever, vomiting, myalgia, flatulence, nausea, anorexia, weight loss, malaise, and fatigue. Some people will have no symptoms at all. Most people who have healthy immune systems will recover without treatment. People with weakened immune systems may develop serious, chronic, and sometimes fatal illness. If any of these symptoms are observed, students/staff will be referred to medical experts for diagnosis (observation of oocysts in stool) and treatment (nitazoxanide).
Members of the lab working with Cryptosporidium will be made aware of the risks and containment protocols necessary to avoid exposure to Cryptosporidium. Everyone will be thoroughly trained before experimentation begins. If lab members think their personal health situation may require additional safety measures, they are encouraged to discuss this with the PI and develop additional safety measures, as is possible, to accommodate their health needs. If at any time a member of the lab has a safety concern (related or unrelated to their health status), they should discuss the concern with the PI. The PI will take appropriate measures to address the safety concern.
Special considerations will be made for those who are pregnant and/or breast feeding, have young children at home, or who are immunodeficient. While risk of exposure and infection is very low, due to the fact that there are currently no efficacious drugs available to treat cryptosporidiosis, it is advisable that pregnant/breast feeding women, those with very young children, or those with immunodeficiencies thoughtfully consider the risks of working with this pathogen. For instances of pregnancy, breast feeding, or having young children at home (<1 yr of age), the following activities wil be conducted on their behalf, as is possible, by other members of the lab: handling infected animals and isolation of Cryptosporidium from infected fecal material (samples >1ml). Often samples can be collected and processed in parallel by the ‘helper’ lab member without much additional effort. In these instances, plans should be made and agreed upon in advance by both members of the lab. Every member of the lab is expected to do their part to ensure everyone’s health is protected, including acting as a ‘helper’ as they are able when requested. Handling small quantities of infected fecal material (samples < 1ml), and activities involving tissue culture pose minimal exposure risks and therefore can be completed by those who are pregnant, breast feeding, or have young children at home.
Members of the lab with immunodeficiences will have the same considerations as those who are pregnant, breast feeding, or who have small children at home. Additionally, members of the lab who have advanced HIV/AIDS, are an organ transplant recipient, or who have a severe immunodeficiency are encouraged to disclose their health requirements to the PI to aid in making an appropriate safety recommendation as to whether or not they can handle Cryptosporidium with minimal risk. This discussion and decision will be made on a case-by-case basis and ultimately the decision will be made by the PI. There may be projects and research activities that do not require handling of Cryptosporidium that may be undertaken by those with severe immunodeficiencies.
Genetic modification of C. parvum has not been shown to increase virulence of the pathogen (as compared to Wild Type). Currently there are no known genetic modifications that can increase the survival of the pathogen in the environment or that impose an increased risk to human health (as compared to Wild Type).

5j. Emergency plan, if required.

Not required. Small scale laboratory work only.

5k. Monitoring

See https://www.lifesci.dundee.ac.uk/services/healthandsafety/other-topics/microorganisms/cl_1_and_2.html#validation

6. Final classification

○ Class 1 ● Class 2 ○ Class 3

7. Additional information