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***Cryptosporidium*: a first step toward tractability**

Lizbeth Hedstrom

Departments of Biology and Chemistry, Brandeis University, MS009, 415 South St., Waltham MA USA 02453

Abstract

The child-killer and famously recalcitrant parasite *Cryptosporidium* is the latest organism to yield to the magic of CRISPR/Cas9. The ability to knockout genes and introduce markers promises a new heyday for drug discovery and vaccine development as well as the basic biology of these fascinating parasites.

Keywords

CRISPR/Cas9; gene knockout; reporter parasites; target validation

Cryptosporidium parasites have traveled a long road to recognition as an important human pathogen. Tyzzer discovered these protozoa at the beginning of the 20th century, believing them to be benign inhabitants of the gastrointestinal tract of most animals (see [1] for a history). It took seventy years to dispel this notion and recognize that *Cryptosporidium* infections cause severe diarrhea, and another thirty years before the recent Global Enteric Multicenter Study (GEMS) revealed that *Cryptosporidium* is the second leading cause of diarrheal mortality in small children after rotavirus [2]. Every year, diarrhea causes the deaths of 800 000 children under five, which is more than that well-recognized child-killer malaria. Alone among the top four diarrheal pathogens, which also include enterotoxigenic *Escherichia coli* and *Shigella* bacteria, no effective treatments or vaccines are on the horizon for *Cryptosporidium* [3,4].

Infection occurs when the spore-like *Cryptosporidium* oocysts are ingested with contaminated water, excyst in the gastrointestinal tract and invade epithelial cells, forming a unique extracytosolic compartment that protrudes into the intestinal lumen (Figure 1). The life cycle involves successive rounds of asexual and sexual multiplication eventually producing oocysts that are excreted. *Cryptosporidium* infections are self-limiting in immunocompetent patients, but can be chronic and fatal in immunosuppressed patients, such as those infected with HIV and in small children, in particular those who are malnourished. While the disease burden is mainly on the developing world, *Cryptosporidium* oocysts are

Corresponding author: Hedstrom, L. (hedstrom@brandeis.edu).

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resistant to commonly used water treatments, so the developed world is also beset by frequent outbreaks. Indeed, *Cryptosporidium* infections account for half of the illness associated with recreational water in the USA [5]. In addition, oocysts can be obtained relatively easily, posing a significant biowarfare threat. The tools to deal with *Cryptosporidium* outbreaks are woefully inadequate. Diagnosis requires specialized tests and treatment is limited to one approved drug, nitazoxanide, that at best it shortens the course of disease by a few days in immunocompetent patients. Perplexingly, *Cryptosporidium* is resistant to antifolates and the many commonly used antiprotozoal drugs. No vaccines are available and no effective treatment exists for immunocompromised patients.

The *Cryptosporidium* field has long been defined by what cannot be done: no continuous culture, inadequate animal models and no genetic tools. *Cryptosporidium* oocysts can be excysted to release sporozoites that infect a mammalian cell monolayer, but undergo just a few rounds of replication before dying. This severely constrains investigation, limiting experiments to small numbers of parasites and a fraction of the life cycle. Parasites can be propagated in immunodeficient animals, though these experiments are largely limited to *C. parvum* rather than the primary human pathogen *C. hominis*. As a result, *Cryptosporidium* drug discovery efforts have been largely futile, with few compounds showing efficacy in animals, much less in humans. Since no truly effective drug exists, we do not even know what pharmacokinetic properties are required to eradicate infections [6].

The genomic sequences of both *C. hominis* and *C. parvum* were reported in 2004, ushering in a molecular era filled with promise [7,8]. The genome revealed that *Cryptosporidium* has numerous transporters but few enzymes. Not surprisingly given its nutrient rich environmental niche, *Cryptosporidium* has very streamlined biosynthetic pathways, instead relying heavily on salvage. Many of the genes appear to have been obtained by horizontal gene transfer from other gut organisms, and thus are very different from other apicomplexan parasites as well as the host [9]. Several promising drug targets emerged, including inosine monophosphate dehydrogenase, calcium dependent protein kinases, hexokinase, lactate dehydrogenase, and fatty acid synthetase. However, target validation required the arduous development of selective inhibitors, always with the fear that the process was not as essential as it appeared on paper. If only there was a way to knockout genes... and now there is!

Using the magic of CRISPR/Cas9, the Striemen laboratory from the University of Georgia now reports a series of elegant experiments resulting in the first gene knockout in *C. parvum* [10]. The team methodically overcame every hurdle, first identifying a construct that transiently expressed nanoluciferase in parasites, then developing a surgical procedure to directly introduce sporozoites into the mouse intestine and finally using CRISPR/Cas9 to introduce aminoglycoside resistance, enabling the selection of transgenic parasites *in vivo*. They exploited an apparent redundancy in thymidine biosynthesis, the presence of both thymidylate synthetase and thymidine kinase (TK), suggesting that perhaps neither gene was essential. Indeed, the TK gene could be disrupted with no apparent consequence-except that the parasite is now sensitive to antifolates!

The availability of nanoluciferase reporter parasites alone is a major step forward for drug discovery, greatly simplifying efficacy and screening assays. The prospects of using genetically encoded reporters to image infections in animals is even more exciting. A screen for essential genes would validate drug targets. The development of attenuated vaccine strains is also on the horizon.

And there is more good news: like its apicomplexan cousin, *Plasmodium* (the malaria parasite), *Cryptosporidium* has sexual stages. But whereas sex requires passage through mosquitos for *Plasmodium*, the entire *Cryptosporidium* life cycle occurs in the mammalian gut, so performing a genetic cross could be as simple as co-infecting strains. With the newfound ability to create parents carrying different genetic markers, isolation of recombinant progeny would be easy. It is no longer completely unimaginable that *Cryptosporidium* could someday become the model apicomplexan of choice.

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Figure 1. A *Cryptosporidium* infected intestine

False color scanning electron micrograph of *Cryptosporidium parvum* showing a group of merozoites emerging from schizonts attached to the epithelial cell surface of a mouse villus (courtesy of David Ferguson, Oxford University).