An inactivated bacterium (paraprobiotic) expressing *Bacillus thuringiensis* Cry5B as a therapeutic for *Ascaris* and *Parascaris* spp. infections in large animals

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**ABSTRACT**

*Ascaris* and *Parascaris* are important parasites in the family Ascarididae, large, ubiquitous intestinal-dwelling nematodes infecting all classes of vertebrates. Parasitic nematode drug resistance in veterinary medicine and drug recalcitrance in human medicine are increasing worldwide, with few if any new therapeutic classes on the horizon. Some of these parasites are zoonotic, e.g., *Ascaris* is passed from humans to pigs and vice versa. The development of new therapies against this family of parasites would have major implications for both human and livestock health. Here we tested the therapeutic ability of a paraprobiotic or dead probiotic that expresses the *Bacillus thuringiensis* Cry5B protein with known anthelmintic properties, against zoonotic *Ascaris suum* and *Parascaris* spp. This paraprobiotic, known as IBaCC, intoxicated *A. suum* larvae in vitro and was highly effective in vivo against intestinal *A. suum* infections in a new mouse model for this parasite. Fermentation was scaled up to 350 l to treat pigs and horses. Single dose Cry5B IBaCC nearly completely cleared *A. suum* infections in pigs. Furthermore, single dose Cry5B IBaCC drove fecal egg counts in *Parascaris*-infected foals to zero, showing at least parity with, and potential superiority to, current efficacy of anthelmintics used against this parasite. Cry5B IBaCC therefore represents a new, paraprobiotic One Health approach towards targeting Ascarididae that is safe, effective, massively scalable, stable, and useful in human and veterinary medicine in both the developed and developing regions of the world.

1. **Introduction**

*Ascaris lumbricoides* or the large intestinal roundworm is the most common nematode parasite of humans, infecting approximately 1 billion people in mostly tropical and subtropical countries [1,2]. These gastrointestinal nematode (GIN) parasites live in the small intestine, reaching more than 30 cm in length. Recent nuclear genome indicated that *A. lumbricoides* and *Ascaris suum*, the ubiquitous and important *Ascaris* parasite of pigs, are highly interbred [3]. Indeed, *A. suum* is a zoonosis that can express as a haplotype of *A. lumbricoides*.
with each parasite species able to infect both pigs and humans [3–8]. In the same family (Ascarididae) and closely related to these Ascaris parasites is Parascaris spp., the omnipresent parasite of foals and young horses around the world that has developed multi-drug resistance, with increasing concern [9]. Ascaris and Ascaris-related parasites are important components of the “One Health” concept [3,10,11].

Infestation in humans with Ascaris is responsible for an estimated loss of 1–1.5 million disability adjusted life-years (DALYs) and for 60,000–200,000 deaths yearly due to obstruction of the digestive tract and hepato-biliary and pancreatic ascaris, the true impact of which has likely been underestimated [1,2,12]. Infection rates can be very high in places, e.g., reaching >50% prevalence in six states in India covering nearly 30% of the total population of India and > 44% prevalence in a systematic meta-analysis of studies conducted in Nigerian children [13,14]. Acute symptoms due to adult parasites inhabiting the gastro-intestinal (GI) tract include abdominal pain, bloating, nausea, vomiting, anorexia, and intermittent diarrhea (with heavy loads leading to serious complications and even death). However, the more significant impact is in children who are more susceptible to these parasites. The consequences of long-term infestation includes malabsorption of nutrients, protein energy malnutrition, and reduced food intake, leading to growth retardation, negative impacts on cognitive development, school absenteeism, and reduced economic productivity [1,2,8,15].

Although single dose anthelmintic cure rates against A. lumbricoides are reported to be >90% [8], there are numerous reports in the literature of deficiencies in treating this parasite with benzimidazoles (anthelmintic of choice for Mass Drug Administration or MDA). These difficulties include lower than expected cure rates with albendazole in Africa and Asia (0%, 19%, 41%, and 56% in four separate studies; [16–19]) and strong persistence of this parasite in African, Asian, and Central American countries despite consistent annual deworming campaigns spanning multiple years [20–25]. Known benzimidazole resistance alleles have been detected in natural populations of Ascaris, although there is evidence that resistance/recalcitrance may occur without these alleles [19,21,26]. Although ivermectin can also be used against this parasite, annual MDA incorporating both albendazole and ivermectin has failed to eliminate it, resulting in only modest prevalence reductions [27].

As noted above, ascars closely related to A. lumbricoides are also common and important parasites of livestock and companion animals. Ascaris suum is a common production and health problem in pigs worldwide because of poor feed utilization and liver condemnation at slaughter where different stringencies of management fail to eliminate infection rates even in large global producing countries [28]. Recent A. suum prevalence surveys in the United States are lacking but routine deworming strategies are used in >77% of production facilities for breeding age pigs and at >17% and >14% for nursery and grower/finisher pigs, respectively [29], indicating a need for practical and effective anthelmintic formulations in the market-place. Parascaris spp. are the most important parasites of foals and young horses around the world [9]. The parasites are truly ubiquitous and virtually every foal should be considered exposed. Equine ascarids cause small intestinal impaction when a cluster of worms block the intestinal lumen. This is a painful and life-threatening condition, and even with successful abdominal surgery, the prognosis for long-term survival is guarded to poor [9]. This is concerning as Parascaris spp. are found widely resistant to currently available anthelmintic drug classes, most notably to the most widely used drug class, the macrocyclic lactones [30]. The pharmaceutical industry has developed and introduced very few new anthelmintic drug classes and none for equine use since the early 1980s, and research exploring alternative treatment modalities is urgently needed. There is real concern that Ascaris spp. could also (independently) develop widespread drug resistance that is now observed in closely related Parascaris spp. Taken together, these findings all point to the urgency and importance of developing new anthelmintic formulations for treating Ascaris and Parascaris parasites of humans, livestock, and companion animals.

Continuing human MDA campaigns and veterinary parasite control strategies would therefore benefit substantially with the addition of new anthelmintics against ascarid parasites with the following requirements: highly and broadly potent against this family of helminths, and safe, inexpensive, and massively scalable for MDA. We have recently described an active pharmaceutical ingredient (API) called IbaCC (see below) containing the Bacillus thuringiensis (Bt) Crystal (Cry) protein Cry5B that is effective against human hookworm infections in rodents and against Haemonchus contortus infections in sheep and that meets all the requirements for MDA [31,32]. Cry5B itself was shown to be active against A. suum in pigs when delivered as a live spore-crystal lysate (SCL) [33]. However, delivery of SCL or of live bacteria in general is not ideal for MDA because of an unstable shelf life, environmental concerns, potential safety/regulatory issues, and development of resistance via suboptimal dosing [31,34–38]. Here, for the first time, we describe and test Cry5B IbaCC against Ascaris infection in pigs and Cry5B itself (as IbaCC) for the first time against Parascaris infection in horses.

2. Materials and methods

2.1. Animal ethics and approvals

All protocols in the study were approved by the University of Kentucky Institutional Animal Care and Use Committee (Protocol 2015–2078) for the equine studies and the USDA Beltsville - Institutional Animal Care and Use Committee #18–008, #18–029 and #17–019 for use of mice and pigs, respectively, and Institutional Biosafety Committee #271.

2.2. Preparation of Cry5B IbaCC and in vitro studies

Cry5B IbaCC was prepared as described [31]. Briefly, spo0A- B. thuringiensis cells expressing Cry5B from a vegetative promoter were cultured in 3×1 LB for 48 h, resuspended in 1/2 volume, treated with essential oil, and then washed. All Cry5B IbaCC used in this study was prepared at the Synthetic Biomanufacturing Facility, Utah State University, at a scale of 350 l. Cry5B concentration was quantitated relative to BSA standards by SDS PAGE densitometry.

Ascaris suum fourth staged larval (L4) parasites were isolated from the small intestines of 86/STAT6KO mice ~12 days post-inoculation (P. L) and shipped overnight to the Araoin laboratory from the Urban laboratory in Beltsville, MD. The next day, the parasites were subjected to in vitro testing with Cry5B IbaCC at two doses and buffer control using the same protocol for setting up and scoring Ascaris larva in vitro [35,39]. In each experiment, each condition contained two wells with five larvae per well. Simply, larvae that moved either on their own or upon touching with a needle were scored as alive and larvae that failed to move even after touching were scored as dead [33,39]. The experiment was repeated twice with two different batches of larvae and two different batches of Cry5B IbaCC. A separate experiment was performed in which Iba (Inactivated Bacterium with empty vector, identical and identically processed to IBaCC in every way except that the vector does not contain a Cry5B insert) was matched in optical density (OD600) to 100 μg/mL IbaCC and tested against A. suum L4 parasites in two independent experiments (n = 20 larvae total). Incubation with Iba resulted in 100% viability (20/20) of A. suum L4 up to and including 96 h incubation.

2.3. Mouse Ascaris in vivo experiment

Breeding pairs of C57BL/6(6B)-STAT6KO were originally procured from the Jackson Laboratories, Bar Harbor, ME and bred at the USDA/ARS/Beltsville Agricultural Research Center in Beltsville, MD. Groups of five mice each containing both males and females were inoculated per os with 10,000 infective A. suum eggs and 12 days later were 1) untreated,
2) treated per os with IBaCC containing Cry5B at 6 mg/kg body weight, or 3) treated per os with IBaCC containing Cry5B at 20 mg/kg body weight. Those in group 3 received a second dose of IBaCC containing Cry5B at 20 mg/kg body weight on the next day. A. suum L4 were isolated from the small intestines six days later by opening the small intestine with scissors and placing them above a tea strainer submerged in 100 ml of phosphate-buffered saline (PBS) maintained for 3 h at 37°C in a water bath. The L4 falling to the bottom of the beaker containing the PBS were then placed in a line-grated rectangular culture dish and counted using a dissecting microscope; larvae were identified morphologically as L4 [40]. These mice were also inoculated with infective Heligmosomoides polygyrus bakeri third-stage larvae and infective Trichuris muris eggs to test Cry5B IBaCC efficacy against multiple nematode infections in a single mouse host. The results of IBaCC treatment on these other parasites will be presented in a separate report.

2.4. Pig Ascaris in vivo experiment

Experimental pigs were obtained from the facility at the Beltsville Agricultural Research Center, Beltsville, MD and were housed and managed as previously described [33]. The pigs were mixed sex and approximately 8 weeks of age with each group containing six pigs at the time of per os inoculation with 10,000 infective A. suum eggs [33]. Pigs were treated per os 12 days after parasitic egg inoculation with a single dose of IBaCC at 30 mg/kg Cry5B body weight (treated group) or a vehicle control (control group) and euthanized four days later to recover A. suum L4 from the small intestine [33]. The L4 isolated from an agar-gel matrix of intestinal contents were counted using a dissecting microscope to determine the number of morphologically identified L4 [40] per pig.

2.5. Horse Parascaris spp. experiment

Ten horse foals born on the University of Kentucky Research Farm in 2019 were enrolled in the study. The foals were exposed to parasites on contaminated pasture to become naturally infected with Parascaris spp. and did not receive anthelmintic treatment prior to the study. From three months of age, the foals were examined for ascarid fecal egg counts determined in triplicate with the Mini-FLOTAC method as previously described [41]. Once foals exceeded 100 ascarid eggs per gram (EPG), they were ranked by egg count magnitude and blocked into pairs of two. From these blocks, foals were randomly allocated to either treatment or control group. Foals in the treatment group were administered a single dose of Cry5B IBaCC at 30 mg/kg via nasogastric tube. Foals in the control group were kept untreated. Egg counts were determined on the day of treatment and weekly for four weeks post treatment.

2.6. Statistical methods

Prism v.8 was used for all graphs and comparisons. The comparison of fecal egg counts in the foal study was carried out using two-way analysis of variance (mixed-effects analysis Time x Treatment). For the in vivo mouse experiment, one-way analysis of variance (ANOVA) with Dunnett’s post-test was used relative to water control. For comparisons including just two groups in the pig study and the end of the horse study, a one-tailed Mann-Whitney (non-parametric) test was used with the assumption that treatment reduced parasite burdens or fecal egg counts. For the comparison of fecal egg counts at the beginning of the horse study, a two-tailed Mann-Whitney test was used to test for equivalence of starting fecal egg counts.

3. Results

3.1. Cry5B IBaCC intoxicates A. suum intestinal fourth larval stage in vitro

Bt Cry5B is a potent anthelmintic protein that has been studied in vivo against hookworms in rodents/dogs and Ascaris infections in pigs delivered as spore crystal lysates or as protein isolated from spore crystal lysates, neither of which are compatible with scale up technologies or broadly effective drug distribution strategies [31,33,42–46]. IBaCC or Inactivated Bacterium with Cytosolic Crystal is an API form compatible with MDA in which Bt Cry protein crystals are produced in the cytosol of asporogenous Bt, that is subsequently killed or inactivated by incubation in food-grade essential oil [31]. IBaCC is effective against hookworm infections in rodents and Haemonchus infections in ruminants (Li et al. 2020; Sanders et al. 2020). A dead probiotic with therapeutic benefit is known as a paraprobiotic [37].

How IBaCC would interact with ascarids, which have different mouth parts, feeding patterns, food sources, and behaviors than hookworms, was unknown. IBaCC containing Cry5B (or Cry5B IBaCC) was initially tested against intestinal A. suum fourth stage larvae (L4s) using a new protocol for isolation from immune deficient mice and incubated in vitro with IBaCC containing two different Cry5B doses. Visible changes in the L4 treated with Cry5B IBaCC relative to buffer control were seen (e.g., paler coloration, degenerated intestinal appearance, rigidity in shape, and reduced movement), indicating clear intoxication of A. suum by Cry5B IBaCC (Fig. 1A). Intoxication was quantitatively evident at 10 μg/mL and more strongly at 100 μg/mL Cry5B content, with near complete death/immobilization seen at 100 μg/mL (~700 nmolar) at 48 h (Fig. 1B). As has been shown with all other parasitic nematodes tested [31,47], intoxication of A. suum by Cry5B IBaCC was dependent upon Cry5B protein since incubation of parasites in the presence of IBaCC lacking Cry5B but otherwise identical to IBaCC resulted in no intoxication (detailed in Materials and Methods). These in vitro data indicated that Cry5B IBaCC, but not the inactivated bacterium B. thuringiensis with empty vector (IBa), has potential to impact A. suum infections in vivo.

3.2. Cry5B IBaCC has a significant impact on intestinal A. suum intestinal infections in mice and pigs

Before testing in large animals, we tested Cry5B IBaCC against intestinal A. suum infections in mice. We have found that C57BL/6J(B6)-STAT6KO mice support the development of A. suum to the L4 intestinal stage, at which point they are expelled (~day 20 post-inoculation or P.I.). B6/STAT6KO mice were inoculated per os with A. suum eggs. Twelve days P.I., the mice (n = 5 per group) were treated with either water (control), IBaCC at 6 mg Cry5B/kg by body weight, or IBaCC at 20 mg Cry5B/kg body weight. Those mice that received 20 mg/kg received a second 20 mg/kg body weight dose day 13 P.I. This double 20 mg/kg dose mimics what was done in the published pig study using Cry5B SCL [33]. Five days later small intestinal worm burdens were determined. Treatment with two 20 mg/kg doses resulted in a statistically significant 92.5% reduction in intestinal worm burdens relative to control (Fig. 2), similar to what was seen in pigs using SCL and the same dosing regimen [33]. Treatment using a single 6 mg/kg body weight of Cry5B in IBaCC also resulted in a significant 62% reduction in intestinal A. suum L4 burdens (Fig. 2). These results in mice with a TH2-immune deficient background further reinforce previous findings that Cry5B efficacy is due to a direct impact on parasites and is not dependent upon an intact immune response [44].

Based on in vitro data and in vivo efficacy in the mouse model, Cry5B IBaCC production was scaled up to 350 L at a contract manufacturing organization (CMO) for studies in large animals [31]. Twelve pigs were inoculated per os with A. suum eggs. Based on success with single dosing in mice, a single-dose treatment trial was performed. Twelve days P.I.,
six pigs were left untreated and six pigs were given per os a single dose of IBaCC at a dose of 30 mg Cry5B/kg body weight. Four days later, intestinal worm counts were determined for all pigs. As shown (Fig. 3), single dose Cry5B IBaCC resulted in a statistically significant reduction and near complete clearance of A. suum L4 from the pig intestine (96% reduction relative to control).

3.3. Cry5B IBaCC clears fecal egg counts in horses infected with the related parasite *Parascaris* spp

*Parascaris* infection is deleterious to equines (foals) and is phylogenetically related to *A. suum* and *A. lumbricoides* [48, 49]. As scaled-up IBaCC effectively cleared *A. suum* infections in pigs at a single dose of 30 mg Cry5B/kg body weight, we similarly tested a single dose of IBaCC at 30 mg Cry5B/kg body weight against adult *Parascaris* spp. infections in naturally infected foals.

Ten foals were selected via rolling enrollment in control (n = 4) and Cry5B IBaCC treatment arms (n = 6). Each foal in the treatment arm was administered via naso-gastric tube a single dose of 30 mg/kg Cry5B in IBaCC produced at 350 l scale by a CMO. Fecal egg counts were determined on the day of treatment and then once weekly for 4 weeks. The results are shown in Fig. 4. Fecal egg counts in foals from both the control and treatment arms were statistically similar at the beginning of the study, with mean ascarid egg counts of 916 and 673 eggs per gram of feces, respectively. Fecal egg counts persisted in all foals in the control group up until and including day 14 and in three out of four foals throughout the entire duration of the study. In contrast, fecal egg counts went to zero in all Cry5B IBaCC-treated foals at the first sample taken one-week post-treatment and stayed at zero for all treated foals throughout the study. Two-way analysis of variance showed a statistically significant difference between control and treatment arms. A significant difference in fecal egg counts at each time point post-treatment was also seen (shown for the final time point; Fig. 4). These data indicated that Cry5B IBaCC had a dramatic impact on *Parascaris* infections in foals.

4. Discussion

Ascarid parasites are some of the most common and damaging...
parasites of humans and animals on earth. In veterinary medicine, the recalcitrance of *Parascaris* to treatment and development to resistance is well documented. Although *Ascaris* infections in humans are considered well managed with current therapeutics, a detailed look at the efficacy data and the propensity for livestock to develop anthelmintic resistance from intensive drug treatment is foreboding for MDA strategies in humans (see Introduction). Therefore, new therapies against this class of parasite in the pipeline are urgently needed and would play an important role in promoting One Health. However, to be therapeutically deployable in both human and veterinary medicine in the developed and developing world, such therapies need to be safe, broadly active against this class of parasite, inexpensive, and massively scalable. We are unaware of any new drugs that meet these criteria that are in the commercial pipeline.

Here we demonstrated that Cry5B IBaCC, a recombinant paraprobiotic (dead bacterial ghost) containing an active Bt Cry protein crystal, has great promise to fulfill this void. Single-dose Cry5B IBaCC is effective against two ascidian species in vivo in three different mammalian hosts. In mice and pigs, Cry5B IBaCC was effective at near complete elimination of *A. suum* parasite burdens in the small intestine. In horses, Cry5B IBaCC completely eliminated parasitic egg counts. Important future studies in horses would include determination of intestinal parasitic burdens in control versus Cry5B IBaCC-treated foals. Because *A. suum* and *A. lumbricoides* are very closely related parasites and because the pig GI tract is considered a good model for the human GI tract, the data suggest that Cry5B IBaCC will be highly effective against *A. lumbricoides* infections in humans. Important future studies would also include testing this hypothesis.

Cry5B IBaCC production was scaled up for these large animal studies to 350 I at a scale at a CMO. Crystal proteins as part of live spore crystal lysates are massively and cheaply produced around the world to control infections in humans. Important future studies would also include testing this hypothesis.

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Cry5B IBaCC treatment has great promise to fulfill this void. Single-dose Cry5B IBaCC is effective against two ascidian species in vivo in three different mammalian hosts. In mice and pigs, Cry5B IBaCC was effective at near complete elimination of *A. suum* parasite burdens in the small intestine. In horses, Cry5B IBaCC completely eliminated parasitic egg counts. Important future studies in horses would include determination of intestinal parasitic burdens in control versus Cry5B IBaCC-treated foals. Because *A. suum* and *A. lumbricoides* are very closely related parasites and because the pig GI tract is considered a good model for the human GI tract, the data suggest that Cry5B IBaCC will be highly effective against *A. lumbricoides* infections in humans. Important future studies would also include testing this hypothesis.

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