I. FINAL REPORT

II. The Use of Egg Yolk Anti-O157:H7 Immunoglobulin to Clear *E. coli* O157:H7 from the Intestinal Tracts of Cattle

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II. Project summary.

The objectives of the research was to evaluate the oral administration of avian anti-O157:H7 immunoglobulin to cattle in order to clear *E. coli* O157:H7 from the intestinal tract. Similar to the principals involved in passive immunity, our hypothesis was that the binding of immunoglobulin to *E. coli* O157:H7 will inhibit motility, attachment, and/or efficient uptake of nutrients and decrease the ability of this bacterium to effectively compete with native microbial flora. Antibodies to *E. coli* O157:H7 are commercially available, but at a $150 per mg, it would cost approximately $7500->$10,000 per dose to achieve our target level of 50-100 μg per ml of rumen fluid in an adult cow. The levels needed for feed and water based administration may be even higher. The production and cost of egg yolk antibody is much less expensive and labor intensive than traditional antibody production practices. The influence of egg yolk anti-O157:H7 antibodies on shedding of *E. coli* O157:H7 shedding in cattle was evaluated by the following objectives:

1.) Immunize chickens with formalin-fixed *E. coli* O157:H7
2.) Harvest and titer anti-O157:H7 immunoglobulin G from eggs
3.) Evaluate immunoglobulin stability in different vehicles (i.e., water, feed, phosphate buffer, egg) and forms (i.e., dry or liquid).
4.) Inoculate steers with *E. coli* O157:H7 and monitor shedding after oral administration of the anti-O157:H7 immunoglobulin. Compare the duration of shedding in untreated (control) steers with those administered immunoglobulin.

5.) **MODIFIED**: Evaluate the influence of chitosan feeding on the shedding of *E. coli* O157:H7 in cattle

III. Project description.

Introduction. A spectrum of illnesses are caused by *Escherichia coli* O157:H7 in humans, ranging from mild diarrhea or hemorrhagic colitis to life threatening hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP; Griffin and Tauxe, 1991). Since first being recognized as a foodborne pathogen in 1982 (Riley et al., 1983), *E. coli* O157:H7 has emerged as a major foodborne pathogen, and epidemiological data indicate that both hemorrhagic colitis and HUS are increasing in number and geographic scope (Griffin and Tauxe, 1991; Griffin, 1995). Ground beef is most frequently linked in foodborne outbreaks of *E. coli* O157:H7 although an increasing variety of foods including apple cider and juice, cantaloupe, fermented sausage, lettuce, mayonnaise, turkey roll, unpasteurized milk, and yogurt have been involved in outbreaks (Alexander et al., 1995; Besser et al., 1993; Griffin and Tauxe, 1991; Mead and Griffin, 1998; Morgan et al., 1993; Padhye and Doyle, 1992; Weagant et al., 1994). In addition to contaminated foods, this pathogen is transmitted by contaminated water, person-to-person, and from animal-to-person (Gouveia et al., 1998; Mead and Griffin, 1998; Renwick et al., 1993; Swerdlow et al., 1992). These latter modes of transmission suggest that *E. coli* O157:H7 has a low infectious dose.

Epidemiological data and surveys indicate that cattle are a reservoir (Garber et al., 1995; Griffin and Tauxe, 1991; Wells et al., 1991), with 7% to 16% of the herds

positive for *E. coli* O157:H7 (Faith et al., 1995; Hancock et al., 1994). Recent data on the prevalence of *E. coli* O157 (H7 and nonmotile) in the feces and on hides of cattle in feedlots was 28% and 11%, respectively (Elder et al., 2000). In a survey of Wisconsin dairy herds, Faith et al. (1995) found 5 of 70 herds (herd prevalence 7.1%) and 10 of 560 weaned calves (animal prevalence 1.8%) tested positive for *E. coli* O157:H7. The differences in prevalence between studies are due to the different ages in the animals examined, the various modes of fecal sample collection and handling, the size of sample tested, and varying detection methods.

Another contributing factor to the variation in prevalence between studies is the sporadic nature of *E. coli* O157:H7 shedding in cattle (Besser et al.,1997; Dargatz et al., 1997; Hancock et al., 1997; Shere et al., 1997; USDA, 1995). Intermittent shedding has been attributed to diet changes (Rassmussen et al., 1993), the lack of sensitivity in sampling and detection methods (Hancock et al., 1994), and the low numbers of *E. coli* O157:H7 found in feces (Besser et al., 1997; Shere et al., 1997). *E. coli* is a minor inhabitant of most gastrointestinal environments (Savage, 1977) and this is true for *E. coli* O157:H7 when present in cattle. Shere et al. (1998) found 10^2 to 10^4 *E. coli* O157:H7 CFU per gram of feces in naturally infected cattle. Cattle shed the organism for varying periods of time ranging from 1 to 16 weeks (Besser et al., 1997; Shere et al., 1998). These results are consistent with inoculation studies that observed shedding in steers from 7 to 14 weeks (Brown et al., 1997; Cray and Moon, 1995; Kaspar et al., unpublished data). These findings raise the question of whether the intestinal tract of cattle is “colonized” during the carrier state. It is possible that *E. coli* O157:H7 is a transient and passes through the bovine intestinal tract into the environment where it can reinfect other animals rather than colonizing the intestinal epithelium. Additionally, inoculation and natural infection of cattle does not prevent future shedding of the same strain of *E. coli* O157:H7 and suggests that immunity has little role in preventing or limiting colonization of the intestinal tract by this bacterium (Cray and Moon, 1995; Shere et al., 1998).

Naturally-infected and inoculated cattle develop serum antibodies to the O157 anitgen but these antibodies do not protect the animal from subsequent shedding. In a study by Hoffman et al. (1997), oral vaccination of calves with 10^{10} CFU of a toxin-negative O157:H7 strain resulted in serum antibodies to the O antigens but did not reduce shedding of the parent O157:H7 strain following inoculation. These investigators also noted that Stx2 had an immunosuppressive effect and suggested that antibodies to Stx2 may be needed to supplement antigens or organisms used as vaccines in order to maximize the immune response. Intimin (encoded for by *eae*) has received attention as a potential vaccine because of its role in adhesion to intestinal epithelial cells (Stewart et al., 1997) and because a majority of strains implicated in human disease produce intimin. However, cattle shed intimin-negative, Shiga-toxin positive *E. coli* at a higher frequency than intimin-positive strains (Sandhu et al.,1996) which raises the question of whether intimin plays any role in cattle carriage. Passive immunity plays an important role in protecting calves from scours caused by enterotoxigenic *E. coli* expressing the K99 pili (Butler and Clarke, 1994). This approach has not been used for *E. coli* O157:H7 and other Shiga-toxin producing *E. coli* because the amount of antibody necessary to protect adult cattle would be cost prohibitive. Antibody production costs can be reduced by the use of vaccinated hens that lay eggs with high titers of specific antibody (ca. 50 mg of immunoglobulin/egg) (Ricke et al., 1988). Given a cost effective source of antibody, passive immunity has the advantage that it avoids the problem of immunosuppression caused by Stx2 (Hoffman et al., 1997). In addition, passive immunity avoids the lengthy process of vaccination that is necessary for protection by humoral immunity. Although the time needed to clear *E. coli* O157:H7 by passive immunity is difficult to predict, inert

particles pass through the intestinal tract of cattle in 2-3 days depending upon the diet. Thus, if the attachment of antibodies block mucosal attachment or render *E. coli* O157:H7 and other Shiga-toxin producing *E. coli* non-competitive, it is reasonable to predict that clearance would occur in 3-7 days.

Rationale and significance. The proposed research addressed one of the objectives of AMIF which is to control *E. coli* O157:H7 in fresh beef products by intervening at the pre-harvest level. Control practices for *E. coli* O157:H7 are primarily applied at the processing level with the most common treatments of carcasses being physical removal of visible fecal contamination, hot water or acid washes, and steam treatment. Despite implementation of these practices, there continue to be a significant number of recalls and beef-associated illnesses associated with *E. coli* O157:H7. A further reduction in the prevalence of *E. coli* O157:H7 in beef will likely require intervention at a different point in the farm-to-consumer continuum. On farm control practices have received little attention although the administration of competitive microorganisms and interruption of waterborne transmission within cattle herds have been proposed as possible strategies to reduce the prevalence and duration of shedding within cattle herds. If the proposed strategy for clearance of *E. coli* O157:H7 is successful, it would be most appropriately applied immediately pre-harvest and complement existing processing treatments to control this important human pathogen.

III. Results

?Initial trial

Laying hens were immunized with formalin-fixed cells of *E. coli* O157:H7 strain 86-24. Eggs were collected from each bird prior to immunization to compare titers of anti-O157 antibodies before and after immunization. Eggs were collected from immunized birds starting one week after the last injection. The titers of anti-O157 immunoglobulins from pre-immunization eggs were <32 and increased significantly following immunization, range 256 to 1024. The average weight of egg yolks was approximately 7 grams and the antibody concentration ranged from ca.1.5 to 6 mg per ml (gram). Thus, the quantity of antibody per egg varied from 10-40 mg per egg. Cattle shedding *E. coli* O157:H7 were fed control eggs (either whole raw egg or freeze dried) or eggs containing anti-O157 antibody either as whole raw egg (5 eggs per day, approximately 100 mg of anti-O157 antibody), or as freeze-dried powder (30 grams of dry egg which constitutes about 375 mg of anti-O157 antibody). The eggs were fed to cattle starting on day 4 post inoculation. The average numbers of *E. coli* O157:H7 shed by cattle fed control eggs or eggs containing anti-O157 antibodies were essentially the same.

?Immunogen

Based upon the results from the initial trial, the immunogen used to immunize chickens was changed. Four *E. coli* O157:H7 strains isolated from cattle were grown in M10 medium (minimal medium based upon intestinal contents) anaerobically. These cells were harvested by centrifugation and lysed using B-per (bacterial protein extraction kit, Pierce). The insoluble fraction of these extracts, which is partly comprised, of outer membrane proteins (OMP) were

pooled and used as an immunogen to inoculate laying hens. A portion of the extracts from each strain was retained and used for protein comparisons by polyacrylamide gel electrophoresis for comparisons of aerobically and anaerobically grown *E. coli* O157:H7. Anaerobically grown cells contained three proteins not evident in aerobically grown cells and another three proteins that were present at greater quantities. These extracts produced titers of anti-O157 antibodies in eggs ranging from 25-50 mg per egg. Based upon the titers of anti-O157 antibody in eggs, the eggs from chickens were split into high, medium, or low categories. Eggs in each category were pooled and freeze-dried. Cattle were randomly assigned to receive control egg or one of the categories of eggs containing anti-O157 antibodies at 40 g per day starting four days after inoculation and continuing to day 9. It is estimated that cattle received daily approximately 500, 300, and 100 mg of anti-O157 egg antibody in the high, medium, and low categories, respectively. Results from this study are shown in Figures 1-3. The average number of *E. coli* O157:H7 shed by cattle receiving the same category of egg antibody and control animals are presented. There was not a significant difference in the number of *E. coli* O157:H7 shed between the control cattle and antibody fed cattle even in cattle receiving eggs with the high-titer of anti-O157 antibodies. However, there appeared to be a trend, or impact, when comparing data from cattle receiving low- to high-titer eggs. The lag before numbers drop was expected considering the transit time through the ruminant intestinal tract is 2-3 days depending upon the diet. Thus, the results suggest that with the use of concentrated or protected antibodies to specific bacterial surface targets, a reduction in the numbers of *E. coli* O157:H7 shed by cattle can be accomplished.

?Year 2

Results from year 1 indicated that the immunogen used to generate egg antibodies was an important aspect to the success of passive immunity reducing the number of *E. coli* O157:H7 shed in cattle. Accordingly, in year 2, there was a focus on the production antibodies to defined targets, such as Tir (receptor for intimin) and outer membrane proteins. In addition, antibody was isolated from eggs by polyethyl glycol precipitation in order to administer high-titer, standardized concentrations, of antibody to cattle.

Secreted proteins from *E. coli* O157:H7 strain ATCC 43895 were used as an immunogen to inoculate laying hens. The choice of immunogen was based upon previously described attachment factors, such as Tir (receptor for intimin), and other secreted proteins that are known to stimulate antibody production in infected humans. The methods employed to immunize laying hens and concentrate antibody worked exceptionally well with over 40 g of antibody to *E. coli* O157:H7 secreted proteins recovered. ELISA was used to quantitate antibody from egg fractions and Western blot analysis confirmed that the antibodies reacted with secreted proteins from *E. coli* O157:H7. Previous results indicated that high-titer antibody preparations (>1g per day) were necessary to impact the numbers of *E. coli* O157:H7 shed likely due to microbial protein

degradation in the rumen. Therefore, antibody was incorporated into chitosan microparticles.

?Chitosan as an antibody carrier for rumen bypass

Antibodies to secreted O157 proteins were administered in chitosan microparticles to inoculated cattle at 2g per day for 5 days while control cattle received chitosan (no antibodies) or no antibody or chitosan (positive control). The feeding of antibodies in chitosan microparticles or chitosan alone resulted in a reduction of 3-4 $\log_{10}$ CFU/g during the period of antibody administration. However, the shedding in control cattle also decreased during this same timeframe. The animal-to-animal variation in shedding made it impossible to conduct any meaningful statistical analysis of the data. This variation was overcome by retaining the persistent shedders for repeated inoculation and testing. This modification enabled us to assess the impact of antibody/chitosan feeding. The results are shown in Figures 4-6.

Figure 4 contains data from animal 43 that was used in three consecutive trial periods. The *E. coli* O157:H7 were allowed to clear from the animal (test negative on four or more consecutive fecal/swab samples) before the start of the next trial. Animal 43 received antibody-chitosan (A) following inoculation (I) in trials one and two. The administration of antibody-chitosan appeared to cause some reduction in fecal shedding when compared to other untreated animals (control) and trail 3 conducted with this animal. However, the average number shed and the average duration of shedding from trials 1 and 2 did not significantly differ from trial 3. The feeding of the antibody microparticles prior to inoculation appeared to depress the level of *E. coli* O157:H7 shed (difference between trials 1 and 2). The antibody microparticles appeared to reduce the numbers and duration of sheeding of *E. coli* O157:H7 in the feces to a greater extent than cells associated with the rectal-anal junction (comparison of fecal and swab samples). These results also indicate that the prior inoculation cow 43 did not protect or significantly alter shedding of the O157:H7 inoculation strain.

Figures 5 and 6 present data from two different animals that were both inoculated without treatment (trial 1), inoculated and feed chitosan [c] only (trial 2), and inoculated without treatment (trial 3). The last trial (number 3) was conducted to address the possibility that immunity was the cause for the decreased number and duration of shedding noted in trial 2. In animal 15 (Figure 5), shedding in trials 1 and 3 was essentially the same although the duration was shorter in trial 3 but the numbers shed were generally higher. The shedding in animal 75 (Figure 6) in trials 1 and 3 was similar. Again, these findings indicate that immunity did not significantly impact shedding of the strain employed and was not the cause of reduced shedding in trial 2. The key observation from this study was that shedding of *E. coli* O157:H7 was significantly reduced in both animals administered chitosan, not detected in animal 15 and detected in only three samples from animal 75.

?Crossover design to assess the influence of chitosan

Based upon the findings made with chitosan feeding, we consulted Peter Crump (UW statistician) to help design an experiment to address the possible influence of chitosan microparticles on *E. coli* O157:H7 shedding. A crossover design was employed to account for the natural animal-to-animal variation in shedding of this organism. The results from this experiment are shown in Table 1 below.

Table 1. Crossover design for evaluating the Influence of chitosan feeding on the shedding of *Escherichia coli* O157:H7 in inoculated holstein bull calves

Animal number	No. of positive samples during the 14 days following inoculation/number of samples							
	CONTROL				CHITOSAN			
	fecal	swab	>103/g or swab	total	fecal	swab	>103/g or swab	total
12	13/13	10/10	16/23	23/23	12/14	12/13	13/27	24/27
13	11/14	9/13	16/27	20/27	2/14	0/13	1/27	2/27
14	11/13	9/10	13/23	20/23	0/14	13/13	0/27	13/27
15	12/14	12/13	9/27	24/27	10/13	8/9	10/22	18/22
16	0/13	1/10	0/23	1/23	8/14	8/13	11/27	16/27
17	10/14	12/13	9/27	22/27	2/13	2/10	0/23	4/23
18	12/14	12/13	11/27	24/27	9/13	6/10	0/23	15/23
22	11/13	5/10	14/23	16/23	9/14	6/13	10/27	15/27
Totals	80/108	70/92	88/200	150/200	52/109	55/94	45/203	107/203
% positive	74	76	44	75	48	59	22	53

Statistical analysis of the above data found that animals fed chitosan had significantly lower numbers of positive samples (total; $p<0.05$) and positive fecal samples ($p<0.05$) than animals fed the control diet. Feeding chitosan did not influence the number of samples containing $>10^3$ CFU/g or swab and the percent of positive swabs of the rectal-anal junction. This data suggests that chitosan interacts with *E. col*i O157:H7 in the lumen rather than cells associated with the rectalanal junction, at least at the concentration tested. In addition, one animal still shedding at the end of this study was administered the remainder of the chitosan (20 g/day) for two days that eliminated detectable *E. coli* O157:H7 in fecal and swab samples by day 3. Future studies need to optimize the level of chitosan feeding in a natural production environment.

?Conclusions

As reported previously, the sporadic nature of shedding and the animal-to-animal variation must be addressed in any study evaluating potential interventions for *E. coli* O157:H7 shedding in cattle. An unknown animal factor (i.e., immune status, microflora, other) that impacts O157 shedding caused us to modify our experimental design to pre-screen animals for shedding patterns before evaluating the impacts of chitosan and chitosan-antibody microparticles. Swab samples of the rectoanal junction yielded results similar to those obtained from

fecal samples. However, colonization of the rectoanal junction was not an indicator of persistent or long-term shedding in cattle because the bacterium is eventually cleared from this site and the intestinal tract in animals housed in a confined environment. Results from this study confirm previous reports that the production of egg antibody is an effective antibody-production method that may be useful in control human pathogens in meat animals by passive immunity strategies. An effective microbial target, immunogen, for antibody production must be known and in the case of *E. coli* O157:H7, antibodies to secreted proteins appeared to have some effect. The administration of antibody to ruminants is complicated somewhat by the protein (antibody) degradation in the rumen and requires that a protective carrier deliver the antibody to the lower intestinal tract. In our tests to use chitosan microparticles as a carrier for O157 antibodies, control animals were observed to have decreased shedding of *E. coli* O157:H7 and led to our crossover study to address the impact of chitosan feeding. Chitosan had a statistically significant effect on *E. coli* O157:H7 shedding which is the major finding from this study. Additional work is needed to evaluate the optimal level of chitosan in feed, the impact of preparation method and form (dry powder vs wet, microparticles, polymer vs monomer, etc.), the effectiveness in field environments, and the influence of chitosan feeding on animal production.

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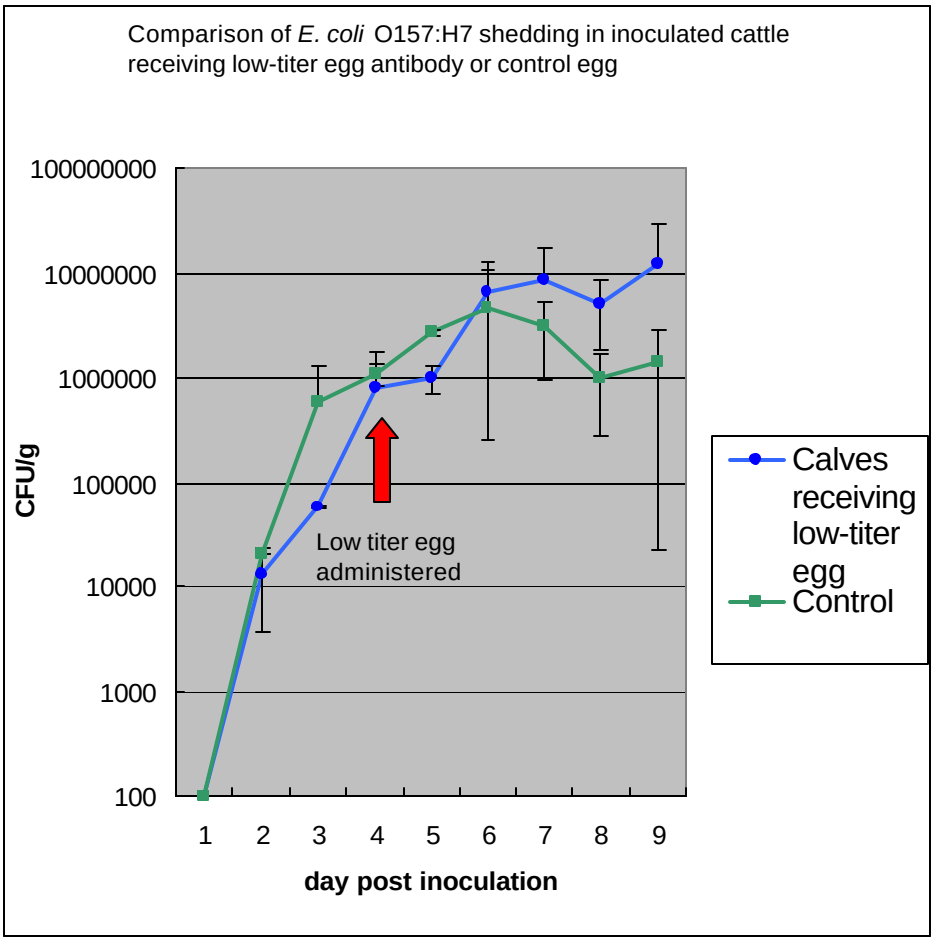
Figure 1


Comparison of E. coli O157:H7 shedding in inoculated cattle receiving low-titer egg antibody or control egg
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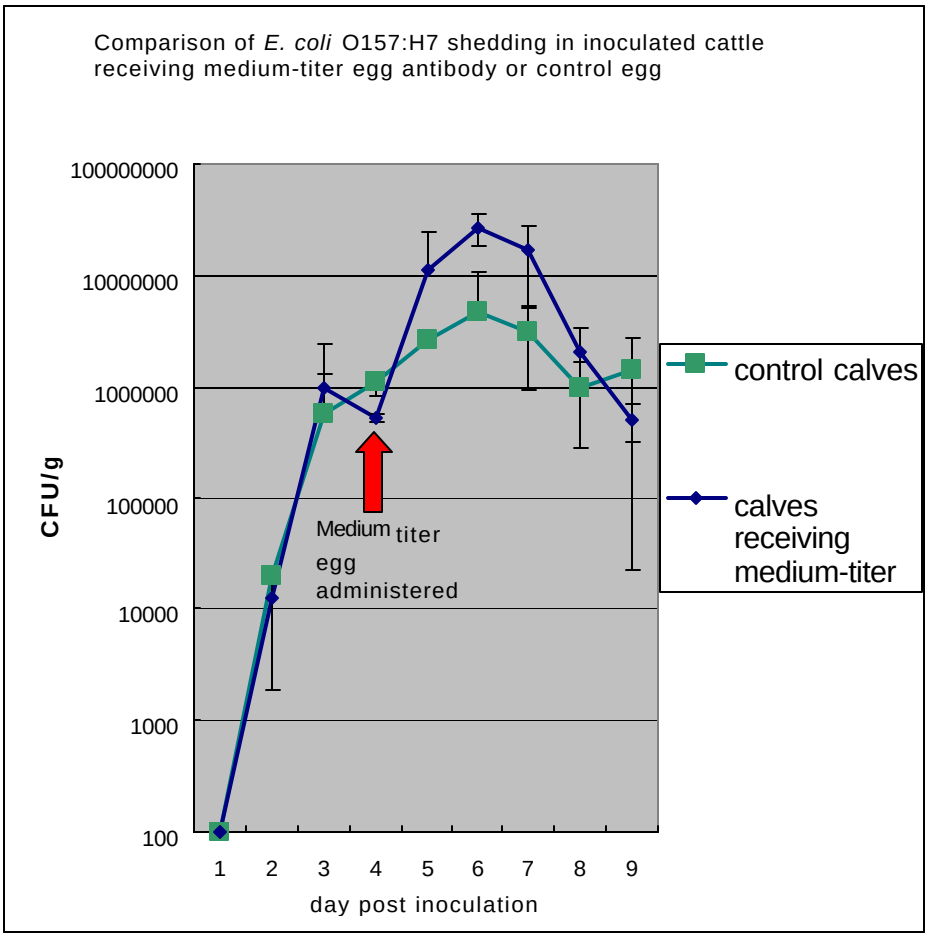
day post inoculation
Low titer egg administered
Calves receiving low-titer egg
Control

Figure 2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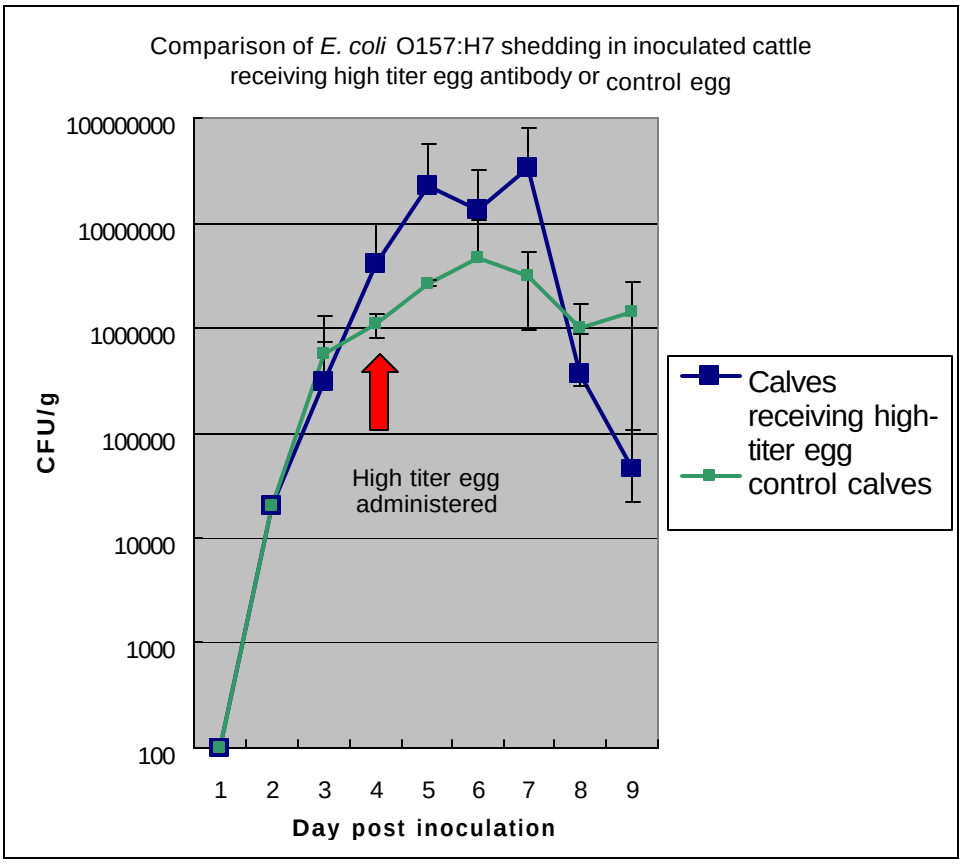
day post inoculation
Medium titer egg administered
control calves
calves receiving medium-titer

Figure 3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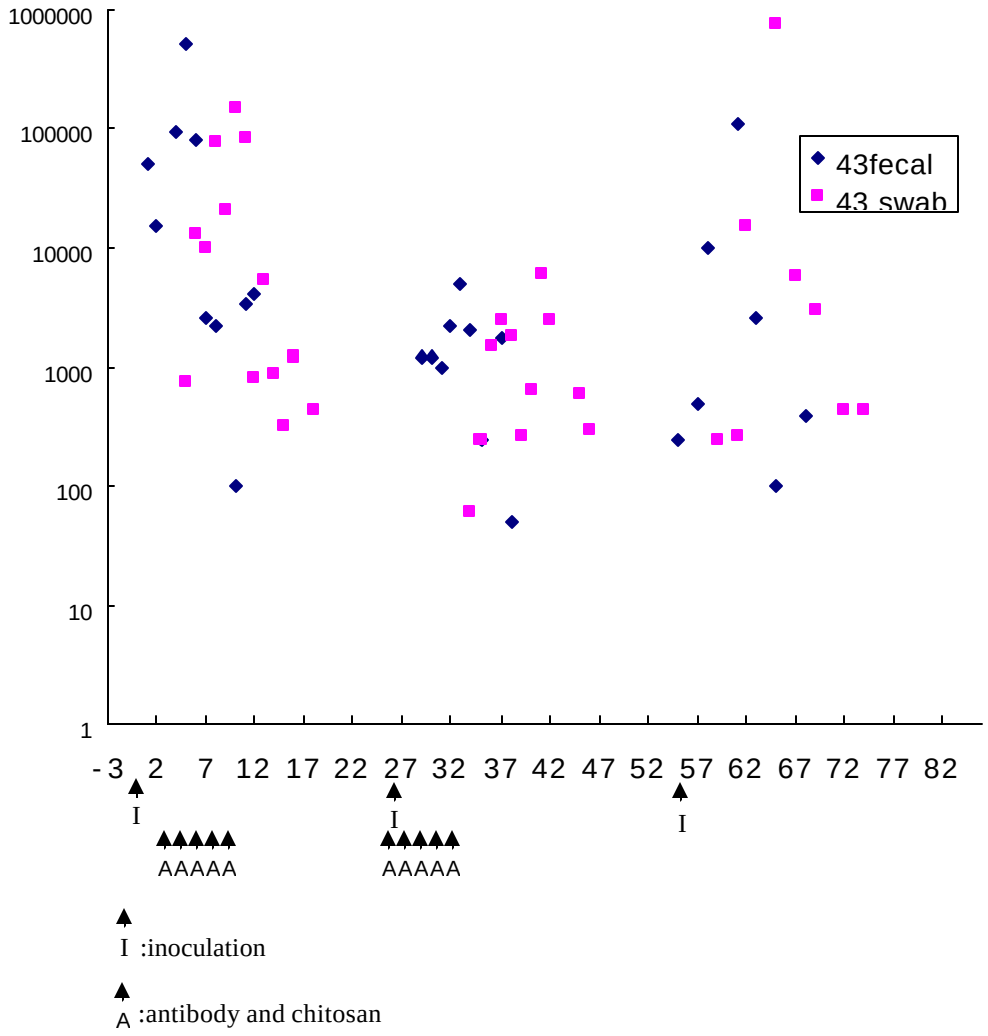
Day post inoculation
Calves receiving high-titer egg
control calves

Figure 4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43 swab
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I :inoculation
A :antibody and chitosan

Figure 5

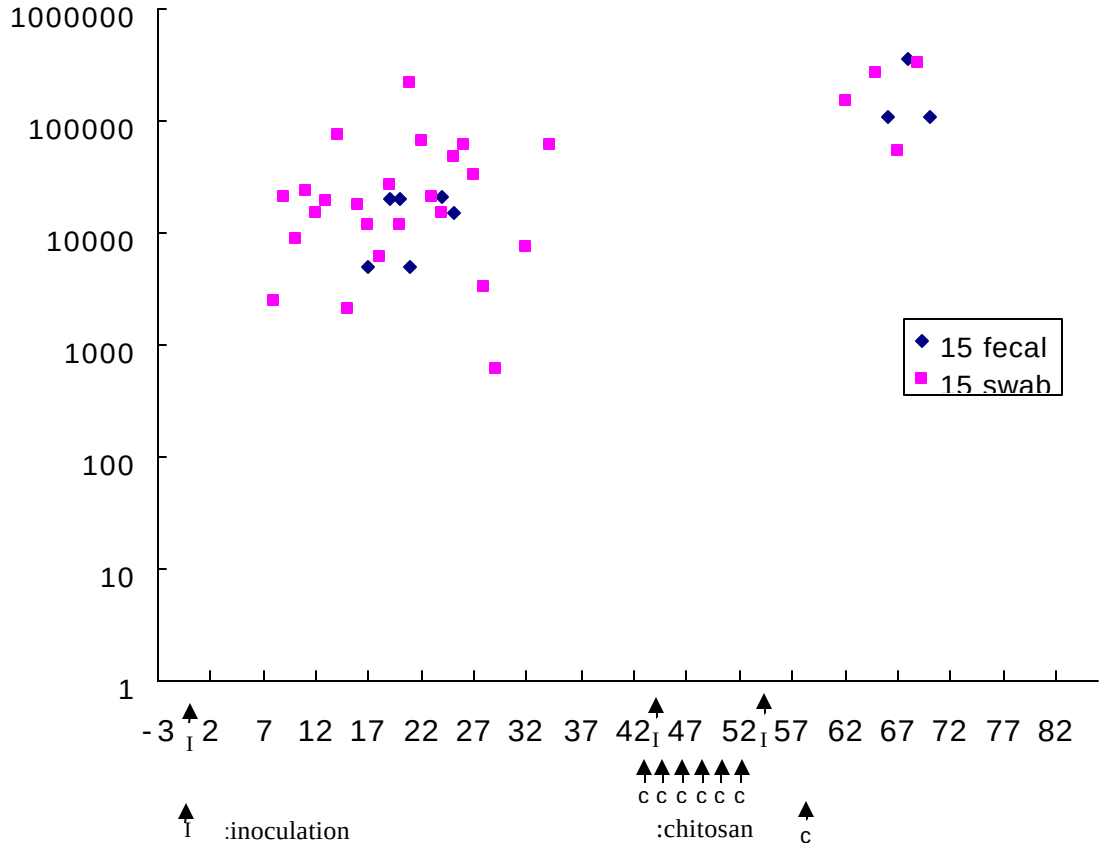
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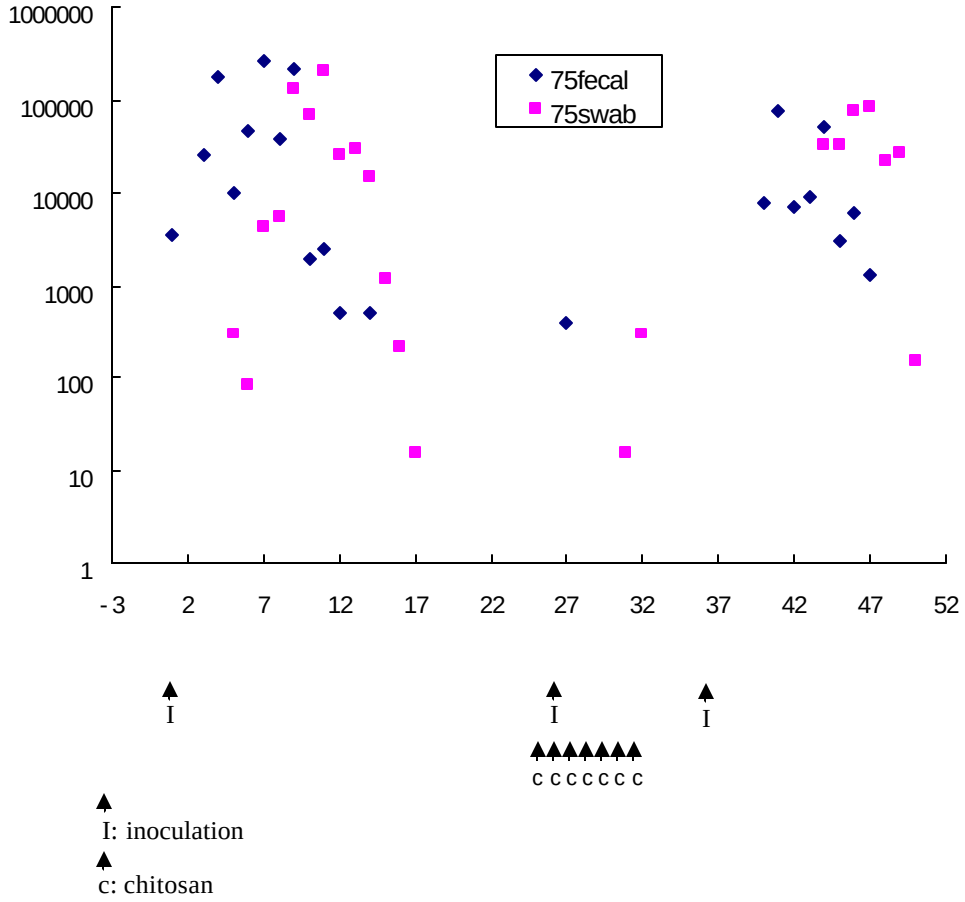
Figure 6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I
c c c c c c c
I: inoculation
c: chitosan