Cain Ken (Orcid ID: 0000-0002-9488-0461)

Title

Comparison of injection and immersion challenges of *Renibacterium salmoninarum* strains in rainbow trout (*Oncorhynchus mykiss*)

Authors

Tifani R. Watson[1,2], Timothy J. Bruce[1,3], Jie Ma[1], and Kenneth D. Cain[1]

Affiliations

[1]Department of Fish and Wildlife Sciences, College of Natural Resources, University of Idaho, Moscow, ID, 83843, USA

[2]Division of Aquaculture, Kentucky State University, Frankfort, KY, 40601, USA (Current Affiliation)

[3]School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, Auburn, AL, 36849, USA (Current Affiliation)

Correspondence

Kenneth D. Cain

Department of Fish and Wildlife Sciences, University of Idaho

Moscow, ID, 83843-1136

E-mail: kcain@uidaho.edu

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Abstract:

Renibacterium salmoninarum is a pathogenic gram-positive bacterium and the causative agent of bacterial kidney disease (BKD), a malady that mainly impacts salmonid species. Experimental challenges were conducted to assess the virulence and challenge route for select *R. salmoninarum* strains (CK-90 and ATCC 33739) in rainbow trout (*Oncorhynchus mykiss).* The CK-90 strain was previously isolated in steelhead trout (*O. mykiss*) from Dworshak National Fish Hatchery (NFH) in 1990. It was intracoelomically injected (100 µL) at a high dose containing 4.80 x 10^6 CFU g $fish^{-1}$ (OD_{525}=1.779) and a low dose containing 6.86 x10^5 CFU g $fish^{-1}$ (OD_{525}= 1.077), or fish were immersed in a solution containing 2.21 x 10^5 CFU g $fish^{-1}$ (OD_{525}=0.886) of *R. salmoninarum*. The ATCC 33739 strain, originating from brook trout (*Salvelinus fontinalis*), was also included and intracoelomically-injected at 3.58 x 10^5 CFU/g (OD_{525}=1.431) to discern differences in virulence between the strains. Clinical signs of BKD manifested approximately ten days post-challenge, and mortalities began 19 days post-challenge. To confirm infection and quantify *R. salmonirarum* antigen load, an enzyme-linked immunosorbent assay (ELISA) was conducted using kidney tissue collected post-challenge. Injection-challenged rainbow trout with CK-90 (both high and low groups) exhibited significantly higher mortality (P<0.001) than fish injected with ATCC 33739 or those exposed to CK-90 via immersion challenge. The *R. salmoninarum* antigen, p57-kDa, was confirmed via ELISA. Antigen load for fish injected with the CK-90 strain (high dose: OD_{405}=0.71, low dose: OD_{405}=0.66) was significantly higher than for fish injected with the ATCC 33739 strain (OD_{405}=0.34). It was found that the CK-90 strain (both high and low doses) was more virulent than ATCC 33739 strain, which exhibited no mortalities over 28 days. Although there were no mortalities during the 28-day trial, the ELISA confirmed that the *R. salmoninarum* antigen

infiltrated kidney tissue in ATCC 33739 fish. Further, the immersion challenge methodology for these *R. salmoninarum* strains was ineffective in inducing mortalities at the abovementioned doses.

Introduction

The bacterium *Renibacterium salmoninarum* has been diagnosed in many countries, and infection with this pathogen can result in disease outbreaks and high mortality (Rozas-Serri et al., 2020). *R. salmoninarum* has often been reported as a chronic infection in farmed salmonid species and causes bacterial kidney disease (BKD), resulting in mortality and economic losses (Chambers et al., 2008; Mitchell and Goodwin, 2004). The pathogen is a Gram-positive diplobacilli that is very slow growing under cool incubation conditions. *R. salmoninarum* may induce an enlarged and swollen kidney within fish; the kidney may contain white granulomatous nodules from bacterial infection (Kaattari and Piganelli, 1996). Further, clinical signs include lesions on the spleen and liver, swollen and fluid-filled abdominal region, and skin petechiation (Sanders and Fryer 1980). Within wild salmonid populations, the pathogen can be transferred horizontally in marine and freshwater environments but only if the infected fish is in close proximity to other fish (Pascho et al., 2002; Murray et al., 2012). *R. salmoninarum* is passed from the infected fish to others by shedding mucus and feces and the bacterium then enters the host through the gastrointestinal tract via ingestion or in an open wound on the surface of the fish (Balfey et al., 1996; Elliott et al., 2015).

Infection with *R. salmoninarum* has historically caused high mortalities for salmonids in the United States. Still, the severity and occurrence of BKD have decreased following various management strategies, including disinfection of eggs with iodophor at 100 parts per million (ppm) for 20 minutes (Yoshimizu 1996), erythromycin feed treatments (100 mg kg body weight^{-1}), erythromycin injection of brood fish, and ELISA-based culling and segregation (Munson et al., 2010). Bruno and Munro (1986) located viable *R. salmoninarum* cells entering the developing oogonia. *R. salmoninarum* was then cultured from both the surface of the egg and

egg contents (Bruno and Munro, 1986). Confirmation of bacterial presence in the ovarian fluid and intra-ovum established vertical transmission as a significant route of transmission for *R. salmoninarum* (Munson et al., 2010).

Although the use of iodophor and antibiotics has decreased infection of *R. salmoninarum*, the risk has not been eliminated via these methods (Gudmundsdottir et al., 2000). The United States has implemented a management strategy that can be used to prevent *R. salmoninarum* transmission in salmon hatcheries. In 1993, Idaho Fish and Game established an ELISA-based culling and segregation rearing method to decrease the risk of both horizontal and vertical transmission of the pathogen (Munson et al. 2010). An ELISA was developed for the detection and identification of *R. salmoninarum* and is a tool widely used to identify *R. salmoninarum* in fish diagnostic laboratories (Dixon, 1987). Brood females have a higher chance of spreading the pathogen horizontally or vertically, whereas male spawners have a limited risk of spreading *R. salmoninarum* (Munson et al. 2010; Fetherman et al. 2020). To determine if a brood female is more likely to transmit *R. salmoninarum* to the eggs, kidney tissue is collected and ran through an ELISA to obtain optical density (OD) levels. OD levels are categorized by negative-lows of OD= <0.25, moderate-highs of OD= 0.25-0.40, and highs of OD>0.40. The eggs are culled if the OD is higher than 0.25 (Munson et al., 2010).

Assessing *in vivo* virulence of *R. salmoninarum* strains in rainbow trout and establishing consistent mortality rates across experiments may aid in further assessing or developing prevention or control methods for BKD for resource-based hatchery programs producing fish for the sport or commercial harvest. Additionally, strains collected across regions may exhibit genetic differences, so more mortality information in a broader array of *R. salmoninarum* collections is beneficial to fish health researchers. It is essential to understand the risks of

introducing fish pathogens through hatchery releases into environments where vulnerable or naïve wild fish populations exist (Fenichel et al., 2009). Due to concerns over *R. salmoninarum* in aquaculture and natural environments, this study aimed to assess the virulence of two different bacterial strains and provide preliminary data for the development of a challenge procedure for *R. salmoninarum* in rainbow trout. Specific objectives for the trial were to 1) determine virulence differences of *R. salmoninarum*, strains ATCC 33739 and CK-90 via injection challenge, and 2) assess the ability of the CK-90 strain to cause mortality via immersion challenge.

Materials and Methods

Rainbow trout used in the study

Healthy juvenile rainbow trout (20.4 ± 0.6 g) were reared in a recirculating aquaculture system at the University of Idaho's Coldwater Laboratory in Moscow, ID. Initially, diploid, mixed-sex eyed eggs were received from Riverence LLC, Rochester, WA, and raised at a 12-14°C. Before study initiation, fish were fed a commercial diet (Skretting classic trout floating pellet, Tooele, UT) at 1-2 % body weight per day.

Fish were transferred from the University of Idaho's Coldwater Laboratory to the University of Idaho's CNR Aquatic Animal Laboratory (AAL) for the BKD challenge. Upon arrival at the AAL (Moscow, ID), fish were split between 14, 190 L experimental tanks with 15 fish in each tank. Fish were allowed to acclimate in tanks for 2 weeks before challenge with *R. salmoninarum*. Tanks were supplied with chilled flow-through, dechlorinated municipal water, and aeration was provided via air stones. No mortalities or clinical signs of illness were found throughout this acclimation period.

Pathogen preparation

R. salmoninarum growth conditions and protocols were based on previous methods, with slight modification (Polinski et al. 2010). CK-90 and ATCC 33739 *R. salmoninarum* lyophilized stocks (stored at -80 ºC at the University of Idaho; both gifted from Dr. Christine Moffit's collection) were rehydrated in 1.5 mL of KDM-2 broth (10 g L^{-1} peptone, 0.5 g L^{-1} yeast extract, 0.5 g L^{-1} cysteine-HCL, pH 6.5) and cultured in 25 mL of broth for approximately 14 days at 15°C, with shaking at 180 RPM. *R. salmoninarum* cultures were then scaled up to 100 mL for an additional 14 days before being scaled to 250 mL and kept at 15°C with shaking at 180 RPM as the final challenge inoculum. The bacterial suspension was also cultured on charcoal agar at each scale-up for 15 days to verify colony morphology (Daly et al. 1985).

At the time of challenge, a 0.5 mL subsample of CK-90 and ATCC 33739 was removed from culture media and placed on a glass slide to confirm the purity of the *R. salmoninarum* suspension. The slide was fixed, Gram-stained, and observed under a light microscope (1000x). This resultant sample was confirmed as gram-positive and contained short, clustered rods characteristic of *R. salmoninarum*. Challenge inoculums were then diluted, and bacterial suspensions were enumerated via hemocytometer counts, according to Polinski et al. (2010).

R. salmoniarium challenge

The virulence of CK-90 was assessed via immersion and injection challenge, whereas fish receiving ATCC 33739 were challenged via intracoelomic injection. An immersion group was not included for the ATCC 33739 treatment as there was limited tank space available in the challenge facility. Each treatment had three replicate tanks (180 L) with 15 fish per tank, with two additional mock-challenged tanks: one tank for immersion treatment and one tank for

injection treatment (unexposed to *R. salmoninarum*; receiving injection or immersion with an equal volume of PBS) for each treatment (14 tanks total). Forty-five fish per treatment group were injected with 100 µL of high (OD_{525}= 1.779; 9.8x10^8 CFU mL^{-1}) or low (OD_{525}= 1.077; 1.4 x10^8 CFU mL^{-1}) doses of *R. salmoninarum* CK-90 based on optical density ranges reported in Polinski et al. (2010). Rainbow trout were anesthetized using 80 mg L^{-1} of buffered MS-222 (Syndel Inc, Ferndale, WA) prior to injection. An additional 45 fish were immersed into a 10L volume (approximately 1:10 (w/v) within 19 L bucket of water with aeration; 90 kg m^3) containing *R. salmoninarum* CK-90 for 1 hour at a dose of 4.5 x 10^7 CFU ml^{-1} (OD=0.886) to determine virulence via immersion delivery. The bath dose was approximated based on a previous immersion challenge reported by Jansson and Ljungberg (1998). ATCC 33739 also had three replicate tanks that were injection-challenged with an inoculum of OD_{525}= 1.431 (7.3x10^7 CFU mL^{-1}).

The temperature during the challenge was 18.1 ± 0.1°C due to seasonal chilling capacity issues, although the 14°C-15°C is deemed the most suitable temperature for *R. salmoninarum* (Jones et al. 2007). Fish were fed daily (to a maximum of approximately 1% of their body weight per day), and any mortalities were collected, and weights recorded. To assess if fish were affected by the bacteria and exhibited clinical signs of disease, all mortalities were necropsied to observe external and internal pathology consistent with symptoms of BKD.

Sampling

Fish were checked daily, and all mortalities were collected for a necropsy and tissue collection to confirm the cause of death via antigen presence (ELISA). Culture via bacterial reisolation was not performed due to the long growth period for *R. salmoniarum*. All clinical signs were

recorded, and the anterior kidney was aseptically collected and placed into sterile, 1.5 mL microcentrifuge tubes, weighed, and stored at -20°C until completion of the trial.

On the completion of the trial (day 28), kidney samples from all surviving fish were collected in 1.5 mL tubes (as described above) for testing using an Enzyme-Linked Immunosorbent Assay (ELISA) test to confirm the presence of *R. salmoninarum* and measure *R. salmoninarum* p57 antigen levels (Mitchell and Goodwin 2004). The protocol used for *R. salmoninarum* detection was from the United States Geological Survey (USGS), and all reagents were manufactured through Kirkegaard and Perry Laboratories Inc. (KPL).

Kidneys were homogenized using a Seward Stomacher 80 (Seward Laboratory Systems, Bohemia, NY). Homogenized kidney tissue was weighed to 0.05g, diluted 1:4 in PBS-T20 for homogenization. The microtiter plates were covered with adhesive seals and incubated for 3 hours at 25˚C. The microplates were then coated with the coating buffer and antibody, sealed with an adhesive plate sealer, and placed in an incubator at 4˚C for 16+ hours. The plates were then washed with a wash buffer solution five times. After washing, the negative control wells were filled with PBS-T20, and the positive control wells were filled with a 1:10, 1:100, and 1:1000 ratio of PBS-T20 of the positive antigen control. Homogenates from the surviving *R. salmoninarum*-challenged rainbow trout. were further diluted 1:25 in PBS-T20.

The microplates were then washed five times to prepare for the HRP-conjugated antibody with milk diluent and sealed for 2 hours at 25 ˚C. After 2 hours, the microplates were rewashed to prepare for the substrate-chromogen and stop solution. Once the substrate-chromogen had been added to the wells, a timer was set for 20 minutes, and the plates were stored in a humid area at 37 ˚C. At 20 minutes, the stopping solution was added to the wells, and results were read with a microplate reader (405 nm) to determine antigen levels from the tissue.

Data analysis

Statistical analysis was conducted using Program R v3.5.1 (R Core Development Team, Vienna, Austria). Trial mortality data were analyzed using a linear regression model and 95% confidence intervals to discern treatment differences, along with secondary verification using contrasts with the Tukey procedure in package "*emmeans*". ELISA values for antigen load in the kidney were analyzed using a Kruskal-Wallis test followed by pairwise comparisons, implementing a Bonferroni correction. α priori was set at 0.05 for all analyses.

Results and Discussion

During necropsies, trout displayed distended coeloms, exophthalmia, dermatitis, blood blisters, and disintegrating skin tissue on the exterior. Internally, the fish had ascites fluid, an enlarged spleen, hemorrhaging on other internal organs, and a swollen kidney with white-grey granulomatous nodules (Dixon 1987). These were all considered typical gross findings of *R. salmoninarum* and were documented accordingly, dependent on the dosage given. Mortalities began approximately 19 days following injection in the CK-90 high dose group (Figure 1). This delayed mortality is due to the slow-growing nature of this bacterium and the selective temperature range. For instance, Rozas-Serri discerned a delay in the mortality curve at 11 °C compared to 15 °C, and in our trial, we used 18 °C, a small interval higher than the optimal temperature (Rozas-Serri et al. 2020). Jones et al. (2007) also noted differences in mortality when juvenile bull trout (*Salvelinus confluentus*) were challenged with a CK-88 isolate of *R. salmoninarum* at 9 °C, and a 10-fold difference in LD_{50} was discerned, compared to 15°C.

Cumulative percent mortality (CPM) for the CK-90 high dose [84.4 ± 4.4 %; 95% CI=0.744-0.945] was significantly higher than the CK-90 low dose [44.4 ± 8.0 %; 95% CI=0.344-0.545]

(P<0.001). Tissue samples collected from mortalities and survivors were tested by ELISA and confirmed positive for both CK-90 (high and low) and ATCC 33739 strains. Treatment group differences were apparent (model adjusted R^2=0.9542; *P*<0.001), with the CK-90 strain exhibiting high virulence (CPM of 84.4 ± 4.4 %), whereas the ATCC 33739 strain did not cause mortality under the challenge conditions (Figure 1), although antigen was present in kidney tissue (Figure 2). The *R. salmoninarum* antigen (p57 protein) load for CK-90 strains and the ATCC 33739 strain was determined by ELISA. Findings confirmed *R. salmoninarum* antigen was present in the fish challenge with CK-90 (high and low groups) with OD_{405}= 0.71 for the CK-90 high dose group and OD_{405}= 0.66 for the CK-90 low dose group, while the ATCC 33739 was OD_{405}= 0.34. These equated to titer concentrations of 3.51 ± 0.00 (-log; exceeding the detection limit for the assay) for both CK-90 groups and 2.18± 0.07 (-log) for ATCC 33739. This was significantly greater than antigen loads of mock-injected fish at OD_{405}= ± 0.06 (*P*=0.020) or ATCC 33739-challenged fish (*P*=0.033). The titer concentrations of the ATCC 33739 strain were 2.18 ± 0.07 (-log) with an OD_{405}= ± 0.34, showing that the ATCC 33739 strain was detected in kidney tissues and the antigen p57 was present (Figure 2). This is interesting, considering the lack of mortality, and suggests that *R. salmoninarum* could establish a carrier state even in fish exposed to non-virulent or virulent strains at low doses (Faisal et al. 2010). This would require further confirmation via cell culture to confirm in future studies, as p57 presence does not constitute bacterial viability. Additionally, the manipulation of dose (increase) or passage of the ATCC 33739 isolate through fish prior to infection, as in Fernandez et al. (1996), may also alter infection dynamics for this strain.

This trial builds upon our understanding of strain variation in *R. salmoninarum* and further evaluates dose-response and mode of delivery for the challenge of rainbow trout. Rainbow trout

were infected with *R. salmoninarum* via intracoelomic injection and immersion to determine virulence differences. The immersion challenge was essential as it mimics how a fish may naturally be exposed to the pathogen, although no mortalities were observed over the 28-day trial. Since *R. salmoninarum* is a slow-growing pathogen, it is possible that it took longer for the pathogen to infiltrate the fish in the immersion trial since it would have to go through the fish's natural defense of mucus and skin (Murray et al. 1992). In contrast, the injection trial bypassed the protective layer causing a more rapid onset of infection. Additionally, Jones et al. (2007) and Jones and Moffitt (2004) demonstrated fish species differences in post-challenge swimming performance and species susceptibility following exposure to isolate CK-88. Therefore, fish strain differences may also be investigated in establishing or comparing experimental BKD challenge models.

Furthermore, since the injection trial bypassed the fish's primary defense mechanism for disease, the infection was manifested efficiently, resulting in the commencement of mortalities at 20 days post-injection and apparent separation between the high and low-dose CK-90 showing at days 22-23 post-challenge initiation. A challenge by Murray et al. (1992) concluded that chinook salmon (*Oncorhynchus tshawytshca*) that were immersed in water containing high and low doses of *R. salmoninarum* (isolate 384) did not exhibit mortalities until day 180 to day 305, depending on the dose given (Murray et al. 1992). Based on this information, fish in the CK-90 immersion challenge may have started showing clinical signs of *R. salmoninarum* past the 28-day trial period. There have been various studies investigating *R. salmoninarum* challenge trial durations, such as 34 days (Campos-Perez et al. 2000), 60 days (Polinski et al., 2010), and 90 days (Jones et al. 2007). Therefore, an extended post-challenge monitoring time may be important to implement in future BKD immersion challenge studies. It is important to note that this preliminary *in vivo*

challenge trial work provided a base dose for the evaluation of strain CK-90 for use in immersion challenges. However, this dose may have still been too low in comparison to the injection challenge doses for CK-90. Modifications for an ideal, elongated period of exposure is also necessary for this strain. Piganelli et al. (1999) performed a 22h exposure, while Campos-Perez (2000), left rainbow trout overnight in 100 L of aerated water. This factor of time (and perhaps an additional mechanism of stress as a factor with static incubations over long durations) also needs to be further defined for this CK-90 stain. Overall, the *R. salmoninarum* antigen, p57, was confirmed via ELISA to have been present in all immersion and injection-challenged trial fish even if no mortalities were recorded during the trial. Results from this trial provide mortality data on the virulence and delivery route of *R. salmoninarum* strains in rainbow trout and establish a baseline for further development of challenge models in cultured rainbow trout. Such efforts are important and may aid in future development and assessment of preventative BKD control methods by incorporating *in vivo* pathogen challenge models.

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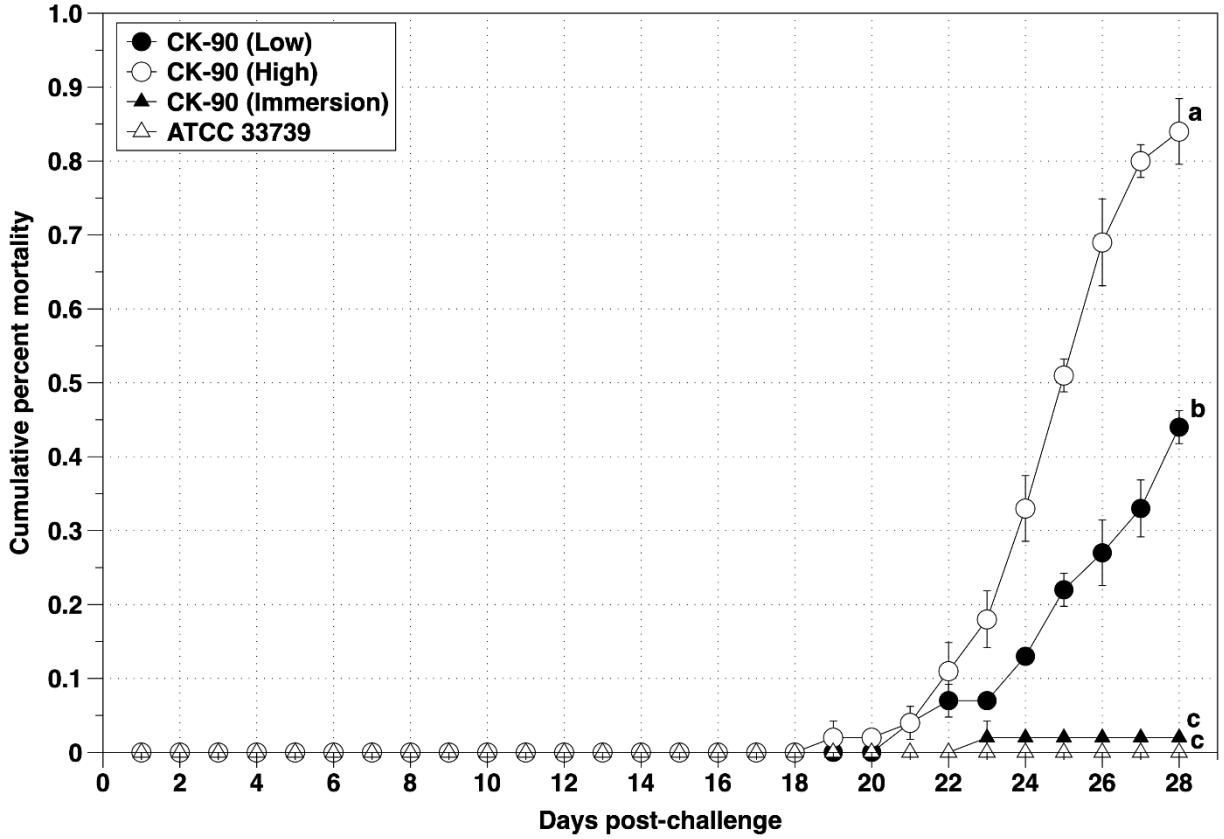
Figure Legends

Figure 1: Cumulative percent mortality of rainbow trout challenged with various strains and doses of *R. salmoninarum*. Presented values are the mean ± SEM (15 fish per tank; n=3). Different letters denote significant differences among *R. salmoninarum* strains used in challenge.

Figure 2: Antigen load (as determined by ELISA) of *R. salmoninarum* in the kidney tissues of challenged rainbow trout at 28 days post-challenge ($P<0.001$). Different letters denote significant differences in antigen load among bacterial strains.



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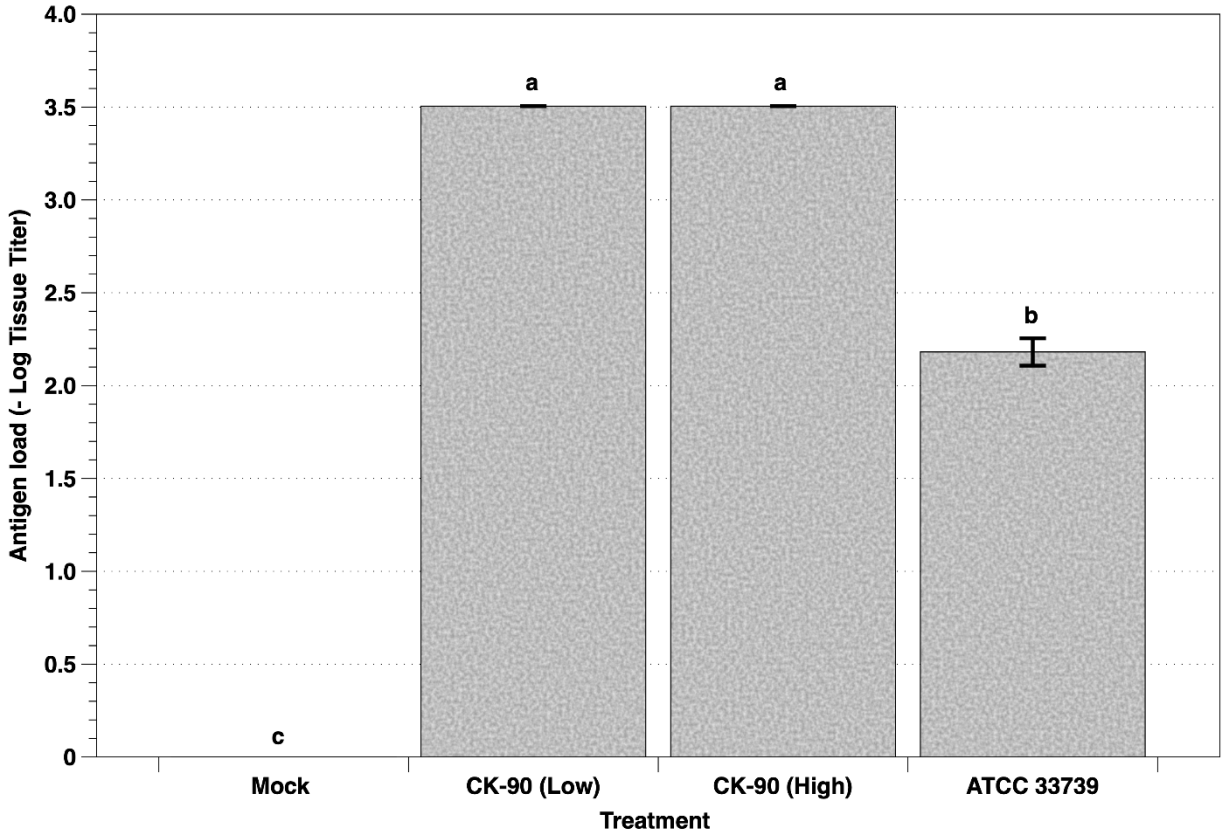

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