STANDARD GUIDELINES FOR THE ELABORATION OF PROTOCOLS AND FOR CONDUCTING BIOASSAY TESTS WITH PISCIRICKETTSIA SALMONIS IN SALMONIDS

This is a working synthesis prepared by Acuaim SpA in order to advise a Consultative Committee and was based on the original Report with the same name prepared by Aquainnovo and Fundación Chile in 2017 for PGSA. The complete Report is available in Spanish and can be requested directly from the Program Staff.

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ABSTRACT

Bioassay tests for pathogens are a fundamental tool for the validation and registration of prod-ucts that have been developed for reducing the effects of piscirickettsiosis. Vaccines, functional diets, chemotherapeutics, genetically selected breeding stock, among others are permanently being studied, and their development and pre-commercial validation requires "in vivo" bioassays for determining their performance in controlled conditions. At present, their design and use by companies, technological centers and universities is not regulated by any minimum standards, affecting the end user's decision-making process given the impossibility of comparing the effectiv-ity of different treatments. In light of this arises the need for generating a minimum standardiza-tion of the methodologies used when conducting bioassay tests for Piscirickettsia salmonis in salmonids in Chile.

INTRODUCTION

Piscirickettsiosis or Septicemia Rickettsial Salmonidae (SRS) is a disease caused by the bacterial agent Piscirickettsia salmonis, and it represents annual losses assessed at approximately 700 million USD, making this pathology one of the main problems for the national salmon industry. The measures for controlling Piscirickettsiosis have been insufficient, including the use of antimicrobials, vaccines, functional diets and genetic selection of fish (QTL SRS). Due to the significant economic impact of SRS in Chile, a high quantity of bioassays of this kind has been carried out in various national research centers. However, Chile is currently lacking a standard protocol that regulates the realization of this type of tests and that defines its fundamental parameters.

The goals of the present paper is to establish guidelines for the realization of standardized bioas-say tests with Piscirickettsia salmonis, to provide a referential baseline containing the minimum requirements necessary for the development of this type of bioassay, to facilitate the review and analysis of the results by governmental entities that regulate the commercial use of these prod-ucts, and to assist in the decision-making process for the salmon-producing companies in Chile regarding the selection of treatments for reducing the productive impacts associated with Piscirickettsia salmonis.

METHODOLOGY

A bibliographical review was carried out in order to establish the state of the art for bioassay tests with P. salmonis. The different aspects of the review took into account the following fundamental aspects: inoculum, fish selection, lethal doses and bioassay tests. At the same time, various steps that must be included in the protocols as minimum requirements were identified, including: a planning phase, an experimental period in which the acclimatization, lethal dose determination and P. salmonis bioassay test execution are included, as well as the closure phase. Lastly, we identified the minimal information that a protocol must include, as well as its structure, the facili-ties, equipment and their technical requirements for the development of a bioassay, and finally the contents that the final report must contain.

RESULTS

Inoculum

A P. salmonis inoculum is defined as a suspension of living microorganisms capable of generat-ing an infection in a host when inoculated. Phylogenetic studies have grouped Chilean P. salm-onis strains into two large groups, EM-90 like (EM-90 strain isolated from Salmo salar) and LF-89 like (LF-89 strain isolated from Oncorhynchus kiutch). Based on this classification, a characteriza-tion of the different Chilean isolates used in experimental bioassays has been done.

Fish selection

The selection must consider aspects such as sanitary conditions, veterinary treatment records and eating behavior prior to acquisition, among other factors. All of these aspects are important for the quality and repeatability of the results of each bioassay. It is important to consider having the fish undergo an acclimation process to the experimental conditions before starting the bioas-say. During this process, the sanitary conditions will be evaluated and the maladjusted or inapt specimens will be discarded from the bioassay process.

Lethal dose

In salmonids, the lethal dose of P. salmonis, as for other bacteria and viruses, is determined by means of a bioassay that consists of inoculating serial dilutions (generally with a factor equal to 10) prepared with virulent suspension of the bacteria. The objective of this bioassay is to deter-mine the correlation between the inoculum titration and the accumulated mortality generated.

Challenge bioassay

This is an experimental test that subjects the fish to inoculation with P. salmonis for a determined period of time. The goal is to determine the efficacy of the tested product or products by means of mortality curves that allow for the comparison of treatments during the realization of the bioas-say. The evaluated treatments must be significantly different, as well as the control groups. It is essential to correctly and promptly register the information generated by the bioassay in order to assure an optimal analysis of the results.

Minimal phases to consider for preparing a bioassay

Planning phase

During this step, the experimental design of the experiment is prepared, as well as the bioassay's protocol, which must be approved by the study's director and by the person responsible for the realization of the bioassay.

Experimental phase

This phase is divided into three sub-phases:

1. Acclimation phase: period in which an organism physiologically adapts to new culture condi-tions. It starts with the introduction of the fish into the experimental center. This includes the evaluation of the sanitary state of the fish, the selection of apt specimens and elimination of maladjusted specimens. The weight and length of the fish, a control record of their feeding, mor-talities and environmental parameters must be registered.

2. Determination of the lethal dose: The goal of this phase is the determination of the titration of the inoculum to be used in the main test. This is done through a bioassay in which serial dilutions of the parental inoculum are inoculated and the accumulated mortality is observed for a defined period of time.

3. Challenge bioassay with P. salmonis: This is divided in two sub-phases: Phase 1) The fish are exposed to the tested product for a period of time defined by the requirements of the developer. Phase 2) Fish inoculation is carried out and the effect of the tested product on the accumulated mortality of the fish is evaluated.

Closure phase

In this phase, the statistical evaluation of the results obtained during the experimental period is carried out and a final bioassay report is prepared.

Minimal information required for an experimental protocol

a. Title: Must be short and informative. It must at least include the specification of the test type, tested product type and the species of fish to be used.

b. Code: All research centers must code their bioassays in order to assure traceability and confidentiality.

c. Personnel responsible for the bioassay: A clear definition of positions and responsibilities for each member of the team responsible for the bioassay.

d. Protocol approval: The protocol must be approved by the study's director and by the person responsible for the realization of the bioassay.

e. Identification and authorizations for the research center: The name of the research center, its geographic location, the resolution number and code given by Subpesca and Sernapesca respectively, authorizing the realization of bioassays with pathogens in salmonid species must be specified.

f. Hypothesis: The working hypotheses must be clearly established.

g. Goals: The general and specific goals must be established and they must answer the working hypotheses.

h. Bioassay justification: The problem and its precedents must be defined, by referencing bibliographical citations that justify the realization of the bioassays.

i. A Gantt chart for the activity: This chart must indicate the starting and ending dates, dates for relevant activities/procedures such as inoculation, weight and length determination as well as sampling, among others.

j. Fish: The species, average objective weight, total number of fish, characteristics and specific requirements (vaccination, pharmacological treatments, sex, stock, etc.) must be indicated.

k. Inoculum: The species of origin, organ, isolation date, isolation location, culture medium, titration of parental inoculum and genovariant must be indicated.

l. Test product: The test product must be generally described and a safety data sheet must be included for the tested product.

m. Experimental design: The experimental design must be described for the acclimation, lethal dose bioassay and challenge bioassay phases, taking into account the volume and number of tanks used, the number of fish per tank, initial culture density, treatment definition, replicas and control groups, feeding and type of food, quantity of required food, description of water quality parameters for the research center, definition of registers and monitoring, photoperiod, sampling frequency and number of fish to be sampled for pathological analyses, duration of the bioassay and definition of statistical models to be used for the data analysis.

n. Letter from the Committee for Animal Bioethics: A letter signed by the Committee for Animal Bioethics must be included, indicating that the experimentation procedures with animals will be carried out using experimental procedures and protocol for the wellbeing of the animals, complying with the ethical norms and bioethical standards described in the "Manual of Bioethical Aspects of Animal Experimentation" from the CONICYT (2009) or another manual of this kind defined by the experimental center.

o. Reports: The number and frequency of the presentation of partial reports must be indicated. Additionally, the estimated date for the presentation of the final report must be indicated.

p. Test product: The test product must be generally described. In this section, a copy of the safety data sheet of the test product must be included.

Required facilities and equipment

Rooms: physical spaces where the bioassays will be carried out.

Tanks: The capacity (useful volume) and the adequate quantity of tanks must be assured in or-der to guarantee the optimal realization of the bioassay.

Water supply system: There must be a constant supply of seawater or freshwater, accordingly, that assures the continuous provision for the different phases of the bioassay.

Sanitary barriers: There must be sanitary barriers in order to avoid potential contamination within rooms as well as to avoid contamination to and from the exterior of the room.

Disinfection system: This must be based on the General Sanitary Program of Influent and Efflu-ent Disinfection Methods and Their Control Modes (PSG AE).

Oxygenation system: It must maintain optimal saturation and dissolved oxygen levels for fish during the course of the bioassay.

Feeding system: This may be automatic or manual.

Illumination system: It must generate an illumination level (4-10 LUX) in accordance with the spe-cific photoperiod for each bioassay.

Operation area: This is the area in which the fish inoculation is carried out. Here, there must be the necessary equipment and supplies for the adequate maintenance and manipulation of both the fish and the inocula.

Storage room: Prepared and delimited area or facility that must possess the specific

environmen-tal conditions for the storage of various products. It must have the appropriate ventilation, be water and humidity-free, have isolation that helps maintain the storage room free of plagues and avoid high temperatures (> 25°C). The experimental center must have storage rooms for storing chemical reagents and food.

Mortality management area: The bioassay rooms or pathogenic units must possess a specific area for carrying out necropsy procedures and tissue sampling. In addition, the bioassay room or pathogenic unit must possess a zone for the denaturalization of mortality, prior to its final disposal.

Electrical back-up system: There must be a system for assuring the autonomy of the system in the case of electrical failure.

Temperature control system: There must be a system that maintains the appropriate water temperature during the realization of the bioassay (boiler, heating pump, chiller, water cooler, etc.).

Human resources: The experimental center must have qualified personnel that assure the correct execution of the bioassay. There must at least be: the main researcher, a veterinary doctor, technical staff and operators.

Measuring equipment: equipment for the regular control of environmental parameters (tempera-ture, salinity, pH, oxygen and other measuring devices) is required.

Biometric measuring equipment: equipment for the measurement of the weight and length of the fish (balances and icythyometers, among others).

Final report

The final report must contain all of the information previously mentioned that is part of the proto-col for the bioassay realization, along with the analysis of the data obtained during the bioassay. In this phase, the raw data from the registers obtained during the realization of the bioassay is complied, in order to organize, summarize and generate statistically analyzed results.

DISCUSSION

It is necessary to validate these results through practical tests in different experimentation centers.

CONCLUSION

The existence of a standardized protocol for the realization of challenge bioassay for P. salmonis will allow for the comparison of different tools for its prevention and control and for the improve-ment of the decision-making process for its end users.


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