Rev Inv Vet Perú 2025; 36(2): e26556
https://doi.org/10.15381/rivep.v36i2.26556

Analysis of antimicrobial sensitivity in *Yersinia ruckeri* strains isolated from *Oncorhynchus mykiss* in fish farms of Ayacucho, Peru

Análisis de sensibilidad antimicrobiana en cepas de *Yersinia ruckeri* aisladas de *Oncorhynchus mykiss* en piscifactorías de Ayacucho, Perú

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Abstract

The cultivation of rainbow trout (*Oncorhynchus mykiss*) has experienced a significant increase in Ayacucho, Perú, leading to a rise of infection diseases, including «red mouth» caused by *Yersinia ruckeri*. This pathogen is typically managed with broad-spectrum bacteriostatic antibiotics, raising concerns about antimicrobial resistance. The primary objective of this investigation was to analyse the antimicrobial sensitivity profiles of *Y. ruckeri* strains isolated from *O. mykiss* in two aquaculture farms located in Vinchos district, Ayacucho. A non-experimental, descriptive cross-sectional design was employed. Ten clinically affected juvenile trout were sampled per farm, and *Y. ruckeri* was isolated using microbiological techniques. Bacterial identification was confirmed through biochemical assays and conventional PCR. Antimicrobial susceptibility testing was performed via the Kirby-Bauer disk diffusion method. It was observed that three strains exhibited resistance to oxytetracycline while one strain showed resistance to florfenicol and chloramphenicol in the first fish farm. In the second farm, four strains demonstrated resistance to tetracycline. Overall, resistance rates to tetracycline and oxytetracycline across both farms were below 30%. These findings highlight emerging resistance patterns in *Y. ruckeri* and underscore the need for prudent antibiotic use in aquaculture to mitigate resistance development.

Keywords: *Yersinia ruckeri*, *Oncorhynchus mykiss*, antimicrobial resistance, red mouth disease, aquaculture, oxytetracycline

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Received: October 27, 2023
Accepted for publication: March 10, 2025
Published: April 30, 2025

Resumen

El cultivo de trucha arcoíris (*Oncorhynchus mykiss*) ha experimentado un aumento significativo en Ayacucho, Perú, lo que ha provocado un aumento de enfermedades infecciosas, incluyendo la «boca roja» causada por *Yersinia ruckeri*. Este patógeno se suele controlar con antibióticos bacteriostáticos de amplio espectro, lo que genera preocupación por la resistencia a los antimicrobianos. El objetivo principal de esta investigación fue analizar los perfiles de sensibilidad antimicrobiana de cepas de *Y. ruckeri* aisladas de *O. mykiss* en dos granjas acuícolas ubicadas en el distrito de Vinchos, Ayacucho. Se empleó un diseño transversal descriptivo no experimental. Se muestrearon diez truchas juveniles clínicamente afectadas por granja y se aisló *Y. ruckeri* mediante técnicas microbiológicas. La identificación bacteriana se confirmó mediante ensayos bioquímicos y PCR convencional. Las pruebas de susceptibilidad antimicrobiana se realizaron mediante el método de difusión en disco de Kirby-Bauer. Se observó que tres cepas mostraron resistencia a la oxitetraciclina, mientras que una cepa mostró resistencia al florfenicol y al cloranfenicol en la primera granja. En la segunda, cuatro cepas mostraron resistencia a la tetraciclina. En general, las tasas de resistencia a la tetraciclina y la oxitetraciclina en ambas granjas fueron inferiores al 30 %. Estos hallazgos resaltan la aparición de patrones de resistencia en *Y. ruckeri* y subrayan la necesidad de un uso prudente de antibióticos en acuicultura para mitigar el desarrollo de resistencia.

Palabras clave: *Yersinia ruckeri*, *Oncorhynchus mykiss*, resistencia antimicrobiana, enfermedad de la boca roja, acuacultura, oxitetraciclina

Introduction

Antimicrobial resistance and its unbridled utilization within the aquaculture sector have assumed paramount significance in global prioritization efforts (FAO, 2021). To ensure the precision of diagnoses and efficacious management of infections among aquatic species, the involvement of specialized professionals is imperative. This participation is vital to uphold the tenets of animal health and adhere to international alimentary trade norms, mandating that each nation should possess pertinent national services dedicated to veterinary oversight and aquatic animal health (FAO, 2009). The global concern for antimicrobial resistance, encompassing antibiotics, transcends multiple domains, including human and animal health, eliciting far-reaching repercussions (OIE, 2009).

The accessibility and deployment of antimicrobials within aquatic organisms are critical factors influencing both animal health and productivity. These elements underscore their pivotal role in ensuring food security, promoting animal well-being, preserving livelihoods, and maintaining the sustainability of animal production systems (FAO, 2020; Luyo Avila, 2020). However, the overuse of antimicrobials can lead to resistance, threatening these benefits (Balta *et al*., 2016). Therefore, sustainable practices and innovation, as emphasized by PNIPA (2019), are essential for balancing productivity with long-term viability. Consequently, a mounting global apprehension revolves around antimicrobial resistance, embracing antibiotics, as they have the potential to offset the merits (FAO, 2009). Notably, between 2011 and 2015, the economic venture of cultivating rainbow trout (*Oncorhynchus mykiss*) in Ayacucho, Peru,

registered an annual escalation rate of 11.5%, rendering it a pivotal driver of the regional economy (PNIPA, 2019).

Yersinia ruckeri, a pathogenic bacterium that infect rainbow trout, engenders the notorious red mouth disease (Sierralta *et al.*, 2013). As a Gram-negative bacterium, it precipitates systemic infections. Its geographical ubiquity is of contemporary relevance (Austin B & Austin D, 2016). This pathogen propagates horizontally through aquatic media, facilitated by the excreta of infected or asymptomatic carriers towards susceptible piscine hosts (Rodgers, 2001). Clinical signs of infection include cutaneous melanisation, abdominal distension, erratic locomotion, languid aquatic surface traversal, and anorexia, especially in the fry stage (Sirvas-Cornejo *et al*., 2011).

Y. ruckeri as one of the most ubiquitous pathogens. Such ubiquity is coexistent with widespread resistance to antibiotics, notably florfenicol, oxytetracycline, ormetoprim-sulfadimethoxine, and trimethoprim-sulfamethoxazole (Ojasanya *et al.*, 2022). Presently, disparate antimicrobial agents are irresponsibly harnessed and subsequently disseminated into the environment, thus engendering the genesis of antibiotic-resistant strains, thereby entailing adverse implications for piscine and human pathogenic entities (Miller & Harbottle, 2018).

In the context of red mouth disease control, antibiotics such as oxytetracycline, sulfadiazine in conjunction with trimethoprim, florfenicol, oxolinic acid, flumequine, and amoxicillin are used (Flores, 2013). Interestingly, in Junín, Perú, *Y. ruckeri*, exemplified by serotype O1 subgroup «A,» showed 100% antibiotic susceptibility amongst evaluated strains (Sierralta *et al*., 2013). In this direction it is important to emphasize accurate diagnoses and effective infection management in *O. mikyss* in the country. The objective of the study was to examine the antimicrobial susceptibility patterns of *Y. ruckeri* strains isolated from *O. mykiss* (rainbow trout) cultivated in two fish farms located in the Vinchos district of the Ayacucho department, Peru.

Materials and Methods

Routine Health Management in Fish Farms

In the two selected farms, routine health management is conducted every 15 days during the dry season and weekly during the rainy season or as needed. Daily removal of dead specimens is performed every morning across all ponds.

Antibiotic Use Practices

Antibiotics are administered upon the appearance of clinical signs of disease. The protocol involves mixing 5 g of antibiotic with 5 kg of feed, supplemented with food-grade oil to enhance medication adhesion. Alternatively, antibiotics are dosed at 5% of the pond's live biomass weight.

A critical factor contributing to non-standardized practices is the limited knowledge among fish farmers regarding the importance of proper antibiotic use. Farmers often medicate based on antibiotic availability rather than established guidelines or sensitivity testing.

Sampling

The sampling method employed, in accordance with the sanitary code for aquatic animals (with clinical signs), followed a specific sampling approach involving the collection of samples from specific or selected sections of the population where the disease's introduction or presence detection is most likely (OIE, 2009).

Collection of Specimens

Samples comprised 10 specimens of rainbow trout per farm, ranging from 8 to 12 cm in length, exhibiting clinical signs such as mouth hemorrhages, fin and abdominal bleeding, skin melanosis, exophthalmia, erratic swimming, and abdominal distension. Fishes were procured from two fish farms located in the Vinchos district, Huamanga province Ayacucho Region, Peru, at an altitude of 3150 meters above sea level. Sampling was conducted during April and May. The specimens were taken to the Microbiology Laboratory of the Faculty of Biological Sciences at the National University of San Cristóbal de Huamanga in polyethylene bags containing a 3:1 ratio of oxygen to water, for immediate processing.

Biological Sample Preparation

For fish sedation and euthanasia, a solution prepared with dechlorinated water and eugenol at a dosage of 100 mg/L was employed. The fish were immersed in the solution, and after 10 seconds, a state of lethargy and reduced fin movement was observed. Once sedation was achieved, euthanasia was performed by severing the spinal cord with sterile scissors. To ensure asepsis, the specimen was disinfected with 70% alcohol and dried using sterile gauze. Precautions were taken throughout the procedure, including the use of latex or nitrile gloves (Ross LG & Ross B, 2008; Noga, 2010).

Necropsy Examination Protocol

The necropsy protocol used in the study was developed by the Laboratory of Ichthyopathology of the Faculty of Veterinary Medicine, National University of San Marcos (2017). In brief, the disinfected fish was placed in a lateral recumbent position on a plastic tray, near a lit Bunsen burner to ensure aseptic conditions. The dissection was carried out using a sterile scalpel and scissors. The organs (kidney and spleen) were identified and examined *in situ*, within the fish's body (Noga, 2010).

Isolation of *Yersinia ruckeri*

Samples from the anterior kidney and spleen were cultured on soy tripticase agar using the surface exhaustion technique and incubated at 22 °C for 24-48 h. Then, colonies resembling those described for *Y. ruckeri* by Furones *et al*. (1993) were selected. These colonies appeared creamy and white, with a diameter of 1 to 3 mm, round, smooth, and with complete edges. Once identified, additional tests, such as Gram staining to determine if they were Gram-positive or Gram-negative, catalase and oxidase tests, were conducted. Distinctive characteristics of colonies suggestive of *Y. ruckeri* were preserved in a stock culture for future research and additional analysis.

DNA Extraction and PCR for Molecular Identification

Genomic DNA extraction was performed at the Y-207 Laboratory of Microbiology and Immunology, Faculty of Biological Sciences, National University of San Cristóbal de Huamanga. The bacterial colony was mixed with sterile physiological saline in a 2 mL tube to reach a McFarland scale of 4. It was then centrifuged for 10 min at 5000 x g (7500 rpm), and the supernatant was discarded. Bacterial DNA was extracted using the Thermo Scientific GeneJET Genomic DNA Purification Kit following the manufacturer's protocol. PCR amplification was carried out in a total volume of 25 µl, containing 10 µl of extracted fish tissue DNA, 0-5 µl of each primer (200 nM), 0.5 µl of Gel track, 1 µL of PCR-grade water and 12.5 µl of AccuStart II PCR ToughMix (Quantabio). The amplification reaction was performed in a thermal cycler, with an initial denaturation cycle for 3 min at 94 °C, followed by 40 amplification cycles, primer hybridization, and chain extension for 40 s at temperatures of 94, 60, and 72 °C, respectively. This was followed by a final extension step at 72 °C for 5 min.

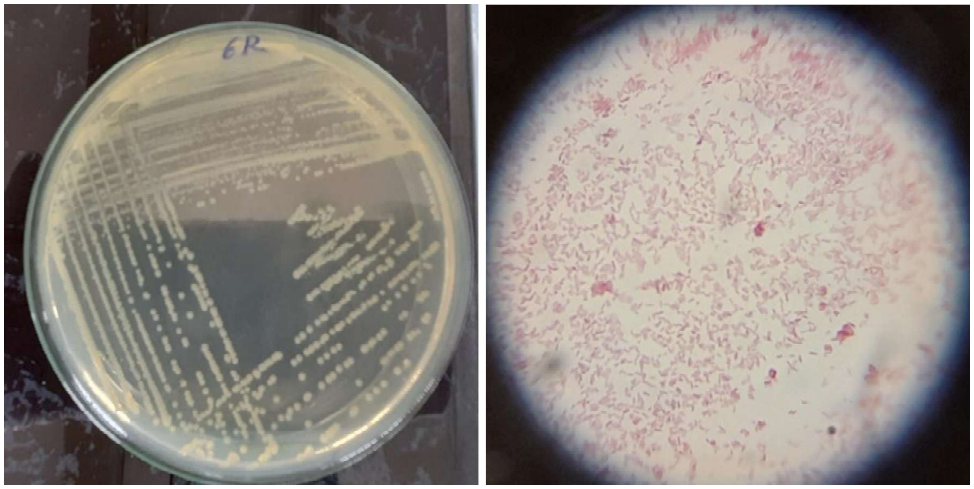

Figure 1. Cultivation of *Yersinia* sp. on Soy Trypticase Agar and microscopic observation

The primers used were YER3 (5'-CGAGGAGGAAGGGTTAAGT-3') and YER4 (5'-AAGGCACCAAGGCATCTCT-3) to amplify a 573 bp amplicon of the 16S ribosomal RNA (rRNA) gene (Gibello *et al*., 1999). In each PCR mixture, negative (no template DNA) and positive controls (50 ng of purified *Y. ruckeri* DNA) were included. The PCR products were resolved on a 0.8% agarose gel. Prior to electrophoresis, 0.5 µL of Xpert Green DNA stain (Grisp) was mixed with 7 µL of each amplicon, and the resulting mixture (7.5 µL total volume) was loaded per well. Electrophoresis was conducted at 75 V for 20 min using the GELATO™ All-in-One Gel Electrophoresis System, which integrates gel running and visualization. Amplified DNA fragments were directly viewed, imaged, and analysed under the system's blue light transilluminator.

Bacterial Sensitivity Testing

Antimicrobial sensitivity was analysed using the Kirby Bauer method for all isolates identified, including oxytetracycline (30 µg), enrofloxacin (5 µg), florfenicol (30 µg), tetracycline (30 µg), oxolinic acid (2 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole (25 µg) by the Kirby Bauer disc diffusion method. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2023).

Data Analysis

Data are presented in frequency tables for antibiotic sensitivity.

Ethical Considerations

The study adhered to the International Ethical Guidelines for Biomedical Research Involving Animals as outlined by the Committee on Ethics and Animal Welfare (Gobierno del Perú, 2012).

Results

Isolation of Yersinia ruckeri suggestive strains

The sampling and isolation of *Yersinia* sp strains from kidney and spleen of clinically ill juvenile *Oncorhynchus mykiss* were done during March to April 2021. Ten samples were collected from each farm. Twenty

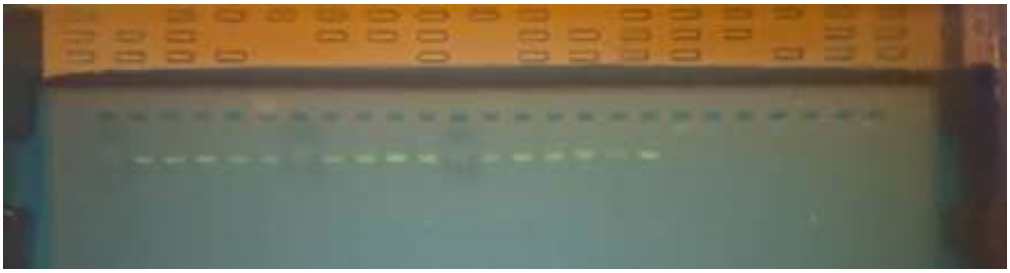

Figure 2. Molecular confirmation of *Yersinia ruckeri* by conventional PCR. Lanes 1, 7, 12: Molecular weight marker (100 bp ladder). Lane 18: Positive control (confirmed *Y. ruckeri* strain). Lane 19: Negative control (no DNA template). Remaining lanes: 573 bp bands corresponding to the 16S rRNA gene amplified with primers YER3/YER4. Electrophoresis was performed on a 0.8% agarose gel and visualized using the GELATO™ system (blue light transilluminator)

isolates of colonies suggestive of *Y. ruckeri* were obtained. These isolates exhibited translucent colonies with regular and convex edges on soy trypticase agar. Gram staining revealed Gram-negative bacteria. In addition, they were positive catalase and negative oxidase (Figure 1).

DNA extraction and PCR for molecular identification

All strains of colonies suggestive of *Y. ruckeri* showed a 573 bp amplification product (Figure 2), while no amplification products were found for any other fish pathogenic bacteria or phylogenetically related bacteria.

Antibacterial sensitivity

The antibacterial sensitivity test of samples (Figure 3) from fish farm 1 revealed that out of the 10 strains, three were resistant to oxytetracycline, and one to florfenicol, tetracycline, and chloramphenicol each. Additionally, nine strains were in the intermediate range for tetracycline, and six for oxytetracycline. All strains were sensitive to oxolinic acid and trimethoprim-sulfamethoxazol.

The antibacterial sensitivity test of samples (Figure 4) from fish farm 2 revealed that out of the 10 strains, only four showed resistances to tetracycline, 10 were in the intermediate range for oxytetracycline, three for florfenicol and tetracycline each. All 10 strains were sensitive to oxolinic acid, chloramphenicol, and trimethoprim-sulfamethoxazol.

Discussion

Yersinia species associated with red mouth disease or yersiniosis was first identified in Peru in *O. mykiss* (rainbow trout) cultures in 2004 (Sierralta *et al*., 2013). In the present study, *Yersinia ruckeri* was identified and characterized molecularly after being isolated from the spleen and kidney of juvenile rainbow trout exhibiting clinical signs of the disease. These findings indicate the presence of this infectious agent in the studied fish farms.

Y. ruckeri affects all stages of the life cycle of *O. mykiss*, with the disease manifesting acutely in fry and chronically in older and larger fish (Wade, 2019). However, in this study, it was prioritized only the juvenile stage because this group exhibited disease characteristics. The bacteria were isolated

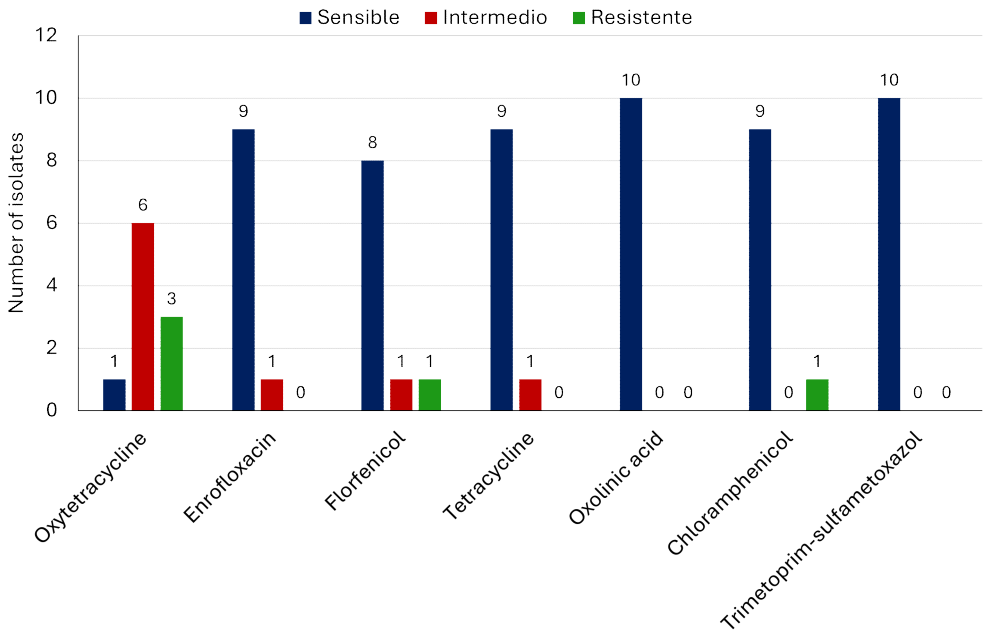

Figure 3. Frequency of antibiotic sensitivity of *Yersinia ruckeri* isolates from *Oncorhynchus mykiss* in fish Farm 1

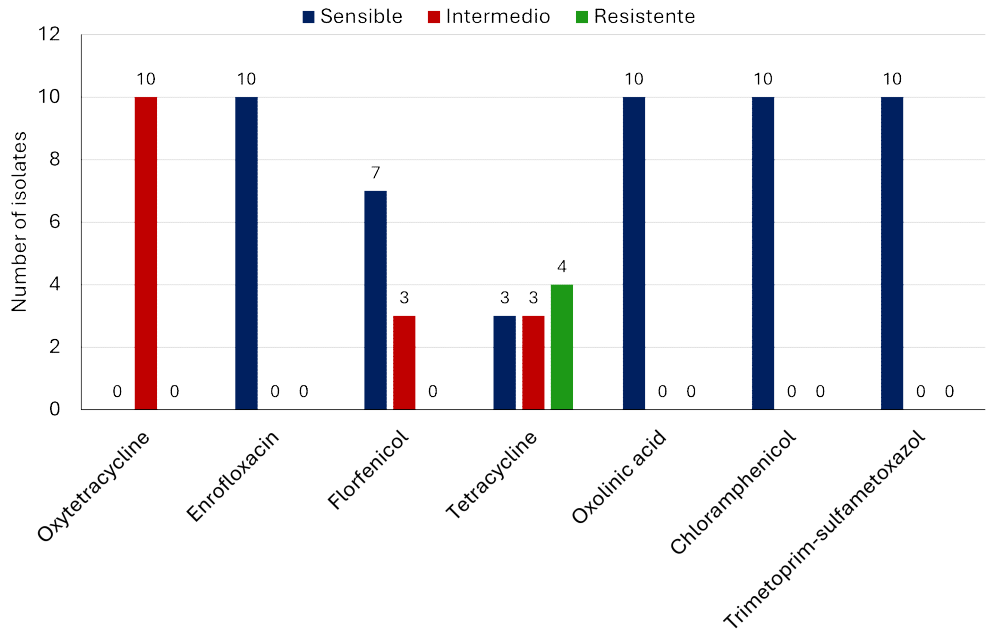

Figure 4. Frequency of antibiotic sensitivity of *Yersinia ruckeri* isolates from *Oncorhynchus mykiss* in fish Farm 2

in autumn (April and May), whereas Huang *et al*. (2015) isolated *Y. ruckeri* in trout farms throughout the year. However, Kayis *et al*. (2009) isolated the pathogen more frequently in the spring and summer in Israel. To obtain more information, a year-long study should be considered.

The characteristics of the colonies after cultivation on soy trypticase agar were creamy white in color, with a diameter of 1 to 3 mm, round, smooth, and with complete edges, compared to the findings of Mesías *et al*. (2019). Gram-negative bacilli, catalase-positive, and oxidase-negative bacteria were observed upon Gram staining, like what was obtained by Balta *et al.* (2016).

PCR consistently resulted in the amplification of the target DNA in all examined tissue samples from trout reported as clinically ill with red mouth disease, as indicated by Gibello *et al*. (1999). The isolation and identification of *Y. ruckeri* species from these samples after selective enrichment suggest that the PCR assay could also be useful for detecting asymptomatic infected fish, which are difficult to identify using traditional bacteriological approaches. However, serotypes present remain to be verified for further studies (Balta *et al*., 2016; Wade, 2019).

This study identified a higher prevalence of tetracycline resistance in *Y. ruckeri* strains compared to other antibiotics, indicating potential limitations in its therapeutic efficacy for managing infections in Ayacucho's trout farms. Specifically, 30% of strains (3/10) from Farm 1 exhibited resistance to oxytetracycline, a drug widely used in aquaculture. This contrasts with findings by Luyo Avila (2020), who reported full susceptibility to oxytetracycline, florfenicol, enrofloxacin, amoxicillin, and sulfamethoxazole/trimethoprim in all 26 *Y. ruckeri* strains analysed in Peru. Such regional disparities may stem from differences in antibiotic application practices, environmental factors, or genetic adaptations within bacterial populations.

The resistance observed in this study aligns with molecular evidence from Balta *et al*. (2016), who linked oxytetracycline resistance in *Y. ruckeri* to the presence of *tetA* and *tetB* genes, which encode efflux pumps that expel tetracycline-class antibiotics from bacterial cells. These findings underscore the need for genotype-phenotype studies in Ayacucho to confirm the role of these or other resistance genes (e.g., *tetC, tetM*) and their implications for treatment failure. To address these challenges, establishing region-specific biosecurity practices is critical. This includes restricting non-therapeutic antibiotic use to minimize selection pressure, implementing routine antimicrobial resistance (AMR) surveillance to guide treatment protocols, and adopting alternatives such as vaccines or probiotics. Additionally, integrating molecular diagnostics—for example, PCR screening for *tet* genes—into AMR monitoring programs could enhance precision in antibiotic selection.

The rising resistance to tetracyclines, despite their historical importance in aquaculture, highlights the urgency of adopting a multifaceted strategy that combines phenotypic susceptibility testing, genetic analysis, and sustainable farm management. Such an approach would not only mitigate resistance but also support the long-term viability of Peru's aquaculture sector.

Regarding antibiotic sensitivity against the disease caused by *Y. ruckeri*, resistance was found in a greater number to tetracycline. In a study by Luyo (2017) all 26 *Y. ruckeri* strains were completely sensitive to oxytetracycline, florfenicol, enrofloxacin, amoxicillin, and sulfamethoxazol/trimethoprim, which differs from the present study where only 3 out of 10 strains were resistant to oxytetracycline in fish farm 1. It is impor-tant to note that Balta *et al*. (2016) found that the gene associated with oxytetracycline resistance through PCR testing is *tetA* and *tetB* genes. These results show that further in-depth studies should be conducted, and effective aquaculture and biosecurity practices should be established.

Conclusions

- *Yersinia ruckeri* strains was successfully identified through a combination of biochemical tests and genetic characterization.
- The strains were isolated from two fish farms located in the Vinchos-Ayacucho district, Peru.
- Antibiotic sensitivity testing revealed varying degrees of resistance to antibiotics among the isolated strains. Resistance was most notable against tetracycline-based antibiotics.

Acknowledgements

Authors would like to express their gratitude to the National University of San Cristóbal de Huamanga, the owners of the fish farms in the Vinchos district, and Nutrimentos JANA S.A.C. for their valuable support and collaboration in this research.

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