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Diagnosis of *Aeromoniasis* in Common Carp Fish by Indirect ELISA Test through Antibody Detection

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Authors' contributions

This work was carried out in collaboration among all authors. Authors FAS and SSNQ conceived and designed the experiment. Authors IJ, HFB and SF conducted and contributed in the smooth running of the experiment. Authors IJ, IQ, BAB and SAD analyzed the data and prepared the manuscript. Authors FAS and FAB reviewed the results. All authors read and approved the final manuscript.

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ABSTRACT

Aeromonas hydrophila, a significant pathogen in aquaculture, poses severe threat to cultured fish species due to its high mortality rates and broad temperature tolerance. Traditional detection methods for *A. hydrophila* are time-consuming, require specific expertise, and are difficult to interpret. In this study, we established an indirect enzyme-linked immunosorbent assay (ELISA) method for the rapid and sensitive detection of *A. hydrophila* using polyclonal antibodies. Fish samples exhibiting clinical symptoms of Aeromoniasis were collected and subjected to microbiological and molecular analysis for pathogen identification. Polyclonal antibodies against *A. hydrophila* were procured and optimized for ELISA. The reaction conditions were meticulously explored through a square matrix titration test to determine the optimal dilutions of antigen and antibody. Results indicated that the ELISA exhibited specificity towards *A. hydrophila*, with a defined cut-off value of 0.2481 for positive samples. The developed assay offers a reliable tool for the early detection of *A. hydrophila* infections in aquaculture, facilitating timely management strategies and mitigating economic losses.

Keywords: Aeromonas hydrophila; ELISA; aquaculture; antibody; pathogen.

1. INTRODUCTION

Fish are extremely susceptible to the multitude of harmful organisms present in their environments because they are constantly submerged in water [1]. *Aeromonas hydrophila* is a deadly pathogen causing mortality outbreaks in variety of cultured fish species [2] and has been identified as one of the most harmful pathogens. With an ideal temperature range of 22-32 ˚C, *A. hydrophila* can survive in temperature range of 0 to 45 °C, which is also its natural habitat in water. According to Semwal [3], *A. hydrophila* is typically regarded as a secondary pathogen that infects fish that have already contracted another infection. Moreover, *A. hydrophila* is an opportunistic intruder that can infect fish when they are stressed or in combination with other infections [4]. Abnormal swimming behaviour at the water surface, reduced feed intake, pale or darker skin with or without ulcer formation, haemorrhage around the mouth, operculum, fin bases, fin erosion, and a swollen belly with hemorrhagic protrusion from the anal opening (dropsy) are all symptoms of the diseased fish [5]. This motile heterotrophic bacterium has curved edges and is shaped like bacilli, but is incapable of producing endospores and is primarily found in temperate zones [6]. An infection with *A. hydrophila* may result from stressful circumstances such as overcrowding, low dissolved oxygen, increased organic content, physical stress, temperature fluctuations, and industrial pollutants [7]. The intensification of aquaculture practices often leads to an increased incidence of disease outbreaks [8,9]. Bacteria can enter fish through physical injuries that are frequently caused by handling, transportation, or aggressive interactions amongst fish. *A. hydrophila* utilizes various virulence factors such as slime formation, haemolysin, proteolytic activity, antimicrobial peptides, enterotoxin, lipolytic activity, aerolysin, cytosine, and gelatinase for protection, survival, and pathogenecity [10]. Seasonal variations and interactions with pathogens affect both the physiology and immune responses of fish [11]. Due to its pathogenic capacity and high mortality rates, motile aeromonas can kill 80-100% of fish within 1-2 weeks, resulting in significant economic losses [12,29-31].

Several diagnostic methods have been developed for the detection of bacterial and viral pathogens [13]. Even though there are many methods for detecting *A. hydrophila*, they utilise an extensive amount of time, require specific knowledge and tools, necessitate careful sample preparation, involve expensive equipment and are vulnerable to intervention from other substances. In the last decade, numerous polymerase chain reaction (PCR) tests have emerged for *A. hydrophila* identification, targeting its virulence gene [14,15] but they are time consuming. Recombinase-aided amplification and loop-mediated isothermal amplification have recently facilitated the advancement of nucleic acid amplification techniques that do not require thermal cyclers [16]. These methods have been utilized to detect infections such as A. hydrophila [17], though they are noted for their low sensitivity [18] and challenging interpretation [19]. One popular way of detection is the serological test and one such method is the indirect ELISA, which is scalable tohandle a high number of serum samples and is effective, sensitive, and specific.In line with this, polyclonal

antibodies were procured against *A. hydrophila* and indirect ELISA method was established. In an indirect ELISA, the target antigen is first immobilized on the microtiter plate, followed by the addition of a primary antibody specific to the antigen, and then an enzyme-conjugated secondary antibody that binds to the primary antibody to amplify the signal [20]. This method is widely used due to its adaptability and amplification power, particularly in complex samples or when conjugating the primary antibody with an enzyme is challenging [21].

2. METHODOLOGY

2.1 Isolation and Identification of Antigen

In this study, thirty Common Carp (*Cyprinus Carpio*) fingerlings showing clinical symptoms of Aeromoniasis were gathered from Dal Lake (Fig. 1). Gill and surface swabs were taken from each specimen, plated on to the nutrient agar having 12 gms/litre of Sodium chloride concentration with pH 7.3 ± 0.1 and incubated at 37°C for 24 hours. The resulting colonies were sub-cultured five times again and again to ensure purity. Colonies displaying morphological traits of *A. hydrophila* (shiny, round and smooth off- white colonies) were further cultured on Rimler Shots Media and again and incubated at 37°C for 24 hours. DNA extraction was performed from the isolates, and PCR was conducted to confirm the presence of the pathogen as per the earlier methodology [22].

2.2 Polyclonal Antibody usedfor Indirect ELISA

Aeromonas hydrophila fkpA unconjugated Polyclonal Antibody against the Immunogen, Recombinant *Aeromonas hydrophila* FKBP-type peptidyl-prolylcis-trans isomerase FkpA protein (21-268aa) raised in rabbit were purchased from Thermofisher (YJ4098408).

2.3 Antibody Optimization

The ELISA procedure was adapted from El-Adawy et al. [23] with slight modifications. Fresh bacterial cultures were centrifuged in 1X Phosphate Buffered Saline (PBS) to attain a concentration of 10^8 cfu/ml. Following this, 100 µl of antigen dilutions were added in ELISA plate using carbonate-bicarbonate buffer (pH 9.6) and the plate was kept at 80˚C for 2 hours, followed by overnight incubation at 4˚C. Dilutions were tested in duplicates. After discarding unbound antigen, the plate was washed thrice with wash buffer (0.05% Tween 20 in PBS) and once with PBS. To block free sites on the wells, a blocking solution (5% skimmed milk powder in PBS) was added and incubated for two hours at room temperature. Subsequently, primary antibodies against *A. hydrophila* were added at varying dilutions and incubated for two hours at room temperature. After rinsing, anti-rabbit antibody at a 1:2000 dilution was added and incubated for one hour. Subsequently, a substrate (5-bromo-4-chloro-3-indolyl phosphate /nitro blue tetrazolium- BCIP/NBT) diluted in the ration of 1:20 with distilled water was added and incubated for ten minutes following three Phosphate-Buffered Saline with Tween 20 (PBST) and oncewith PBS. The reaction was stopped by adding 50 µl of 1M H_2SO_4. Optical density (OD) at 450 nm was measured using an Epoch Biotek ELISA reader.

2.4 Determination of cut-off and Specificity of Developed Indirect ELISA

To establish the cut-off values for positive and negative samples, 30negativefish samples devoid of *A. hydrophila* were analyzed using the indirect ELISA method. To assess the specificity of the indirect ELISA, antibodies to *Flavobacterium, Staphylococcus,* and *Pseudomonas* were employed as samples and subjected to the established protocols.

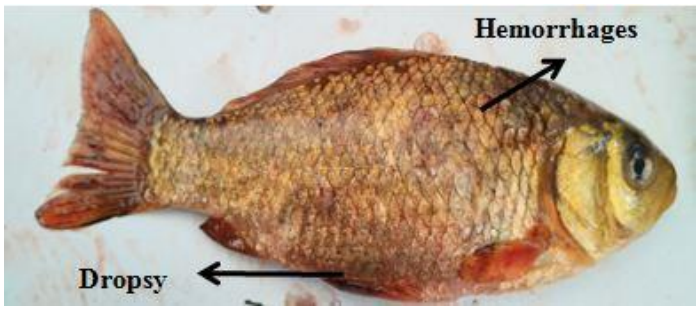

Fig. 1. *Aeromonas* infected samples of Common Carp

3. RESULTS

As we aim to determine the optimal reaction conditions,we embarked on an extensive square matrix titration test, meticulously exploring various combinations and concentrations (Table 1). Our goal was to discern the conditions that would yield the most favourable signal-to-noise (P/N) values, indicating optimal performance. As our data analysis unfolded, it became evident that the conditions associated with the highest P/N ratios consistently emerged as the most promising. In Table 1, these paramount conditions are prominently highlighted. Specifically, our investigation unveiled that the optimal dilution for the coating antigen stood at 1:500 of 6×10^8cfu/ml (1.2×10^6cfu/ml), a concentration that exhibited remarkable efficacy. Additionally, our findings pinpointed the optimal working dilution for the polyclonal antibody at 1:1600, underscoring its efficacy in the intended application.

3.1 Cut-off Values Obtained

The obtained results revealed an average OD_{450} value of 0.2481 with a standard deviation of 0.0136 (Fig. 2). Consequently, samples with OD_{450} values equal to or greater than 0.2481 were classified as positive, while those below this threshold were deemed negative.

3.2 Specificity of Developed Assay

During the specificity test, positive reactions for *A. hydrophila* positive samples exhibited values exceeding 2.606, whereas negative reactions fell below this threshold (Fig. 3).

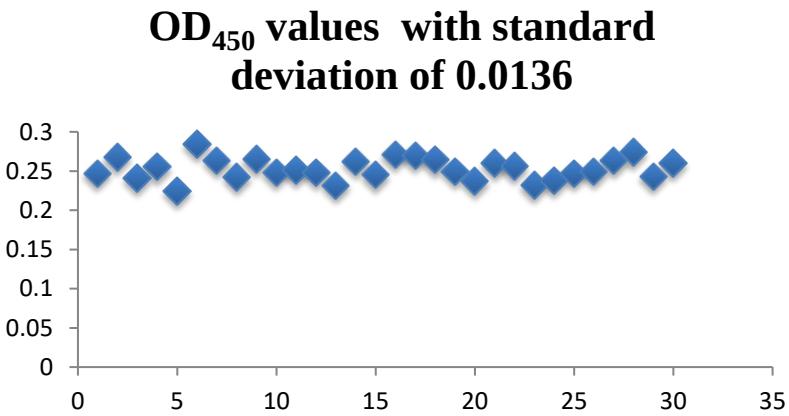

Fig. 2. Establishing the cut-off value for the indirect ELISA involved calculating the mean OD value, resulting in a determined cut-off threshold of 0.2481

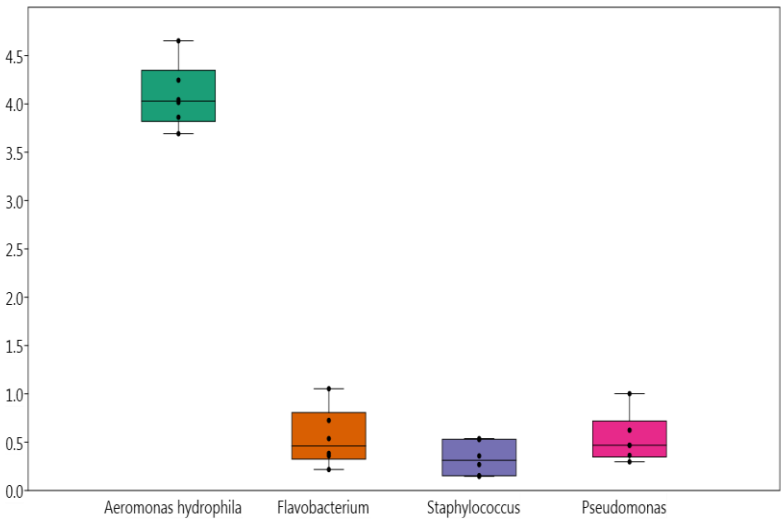

Fig. 3. Specificity assessment of indirect ELISA

Table 1. P/N ratios obtained from the square matrix titration test of the antigen-antibody indirect ELISA; a) With duplicates b) Average

a) With duplicates

Primary Antibody	Antigen Dilutions							
	1:250 of 6 ×10^8cfu/ml		1:500 of 6 ×10^8cfu/ml		1:750 of 6 ×10^8cfu/ml		1:1000 of 6 ×10^8cfu/ml	
1:200	2.519	2.693	3.028	3.132	3.219	3.198	3.095	2.971
1:400	3.017	3.057	3.213	3.263	3.107	3.128	2.853	3.019
1:800	3.381	3.375	4.579	4.493	3.901	3.972	3.279	3.294
1:1600	4.312	4.376	4.816	4.974	4.602	4.691	3.397	3.419
1:3200	3.572	3.691	3.419	3.483	3.726	3.819	3.176	3.193

b) Average

Primary Antibody	Antigen Dilutions			
	1:250 of 6 ×10^8cfu/ml	1:500 of 6 ×10^8cfu/ml	1:750 of 6 ×10^8cfu/ml	1:1000 of 6 ×10^8cfu/ml
1:200	2.606	3.08	3.208	3.033
1:400	3.037	3.238	3.117	2.936
1:800	3.378	4.536	3.936	3.286
1:1600	4.344	**4.895**	4.646	3.408
1:3200	3.631	3.451	3.772	3.184

4. DISCUSSION

The pathogen ecology and disease process are determined by the epidemiological triad, which includes the host, environment, and pathogen interacting [24]. The development of an indirect ELISA for the rapid and sensitive detection of *A. hydrophila* in aquaculture is a significant contribution to disease management strategies. This assay addresses the pressing need for efficient diagnostic tools to mitigate the devastating impacts of *A. hydrophila* infections on fish populations. One of the notable strengths of the ELISA method lies in its sensitivity and specificity [25], which are essential for accurate pathogen detection. Through meticulous optimization of reaction conditions and careful determination of cut-off values, the assay demonstrates high specificity towards *A. hydrophila*, minimizing the risk of false-positive results. This specificity is crucial for distinguishing between infected and non-infected specimens, allowing for precise disease surveillance and control.

Our investigation revealed that the ideal dilution for the coating antigen was determined to be 1:500 of6 x10^8cfu/ml which is equivalent to 1.2 x10^6cfu/ml, a concentration that demonstrated exceptional efficacy. Earlier scientists have developed ELISA to detect *Pasteurella piscicida* in culture and in 'spiked' fish tissue [26]. The sensitivity threshold for detecting bacteria in culture was found to be 10^3 cells per milliliter or 10^2 cells per well if a plate reader is utilized, or 10^5 cells per milliliter (10^4 cells per well) through visual inspection. Comparable sensitivity levels were attained, 10^3 cells per well with a plate reader and10^4 cells per well visually, when conducting ELISA on tissue samples. Similarly, MacPhee et al. [27] developed an ELISA to estimate the quantity of *Flavobacterium branchiophilum* on the gills of rainbow trout *Oncorhynchus mykiss*. The detection limit was approximately 1 x 10^3 bacteria per milliliter, with mean intra-assay and inter-assay variabilities of 6.7% and 8.1%,respectively, during routine utilization. ELISA absorbance at 405 nm corresponded directly to the quantity of *F. branchiophilum* present across a range of antigen concentrations from 0 to 80,000 cells per milliliter. This held true whether testing whole bacterial cell preparations, gill preparations spiked with bacterial cells, or extracts from infected gills. Following the systematic evaluation and refinement of detection parameters. Chen et al. [28], established the most effective concentrations for the coated-bacterial antigen, polyclonal antibody, and enzyme-labeled secondary antibody, which were determined to be 1:800 (2.99x10^7 CFU/ml), 1:6400, and 1:5000,respectively while developing and evaluating indirect ELISA for *Klebsiella pneumonia*. Additionally, they identified optimal

conditions for coating and blocking, both conducted at 4°C for 12 hours, The preferred dilution buffers for the bacterial antigen, antibodies, and blocking buffer were 0.05 mol/L carbonate buffer, phosphate buffer with 1% BSA, and carbonate buffer with 1.5% BSA, respectively. In present study, the optimal working dilution for the polyclonal antibody was found to be 1:1600.

The validation of the ELISA method through specificity assessment and determination of cut-off values enhances its reliability and utility in practical applications. By establishing defined criteria for positive and negative samples, the assay provides a standardized approach to pathogen detection, ensuring consistent and reproducible results. The current study revealed an average OD_{450} Value of 0.2481 with a standard deviation of 0.0136 as the cut-off values. Innovative serological diagnostic method employing nano-silver ELISA for swift detection of *Aeromonas veronii* infections in Nile tilapia was developed by El-Adawy et al. [23]. Identification of *A. veronii* isolates was conducted through alignment analysis of gyrB and 16S rRNA gene sequences. The established cut-off values for traditional and nano-based ELISA were determined to be 0.46 and 0.48, respectively. Earlier, the cut-off value was established as 0.28, with an analytical sensitivity of 1:800 by Chen et al. [28] for *Klebsiella pneumonia*. No instances of cross-reactions observed with other bacterial isolates. In the current study, positive reactions for *A. hydrophila* positive samples, were indicated by values surpassing2.606, while negative reactions; *Flavobacterium, Staphylococcus,* and *Pseudomonas* registered below this threshold during specificity testing. In developed ELISA for swift detection of *A. veronii* infections in Nile tilapia, there was no cross rection with *Aeromonas hydrophila, Aeromonas caviae, Aeromonas sobria, Pseudomonas fluorescens,* and *Vibrio vulnificus* [23].

While the ELISA method offers numerous advantages, it is essential to acknowledge potential limitations and areas for improvement. One such limitation is the need for specialized equipment and expertise to perform the assay accurately. However, advancements in technology and training programs may address this limitation, making the method more accessible to aquaculture practitioners. Additionally, future research should focus on various ways of optimizing the assay protocol in order to enhance its sensitivity, efficiency and productivity, thereby expanding its applicability in diverse aquaculture settings.

5. CONCLUSION

The establishment of an indirect ELISA method for the rapid and sensitive detection of *A. hydrophila* using polyclonal antibodies represents a significant advancement in aquaculture disease management. Through meticulous optimization of reaction conditions and careful determination of cut-off values, this assay demonstrates specificity towards *A. hydrophila*, offering a reliable tool for early detection in infected fish populations. Moreover, the scalability and practicality of the ELISA method make it a valuable asset for on-site use in aquaculture facilities, facilitating proactive disease surveillance and control measures. Overall, this study contributes to enhancing the sustainability and productivity of aquaculture by addressing the challenges posed by *A. hydrophila* infections.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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