

# Development of Co-agglutination Kit for the Diagnosis of *Pseudomonas fluorescens* Infection in Fishes

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## Abstract

Among the *Pseudomonads*, *P. fluorescens* is a dominant component of the freshwater ecosystem and its infection is widespread in aquaculture. In the present study, it was envisioned to develop and standardize an immunoassay kit for monitoring and detecting *P. fluorescens*. A co-agglutination test using protein A from *Staphylococcus aureus* (Cowans I) strain that is simple to operate, on-site and non-laboratory was standardized for the detection and quantitative determination of *P. fluorescens* infection. A 10% solution of *S. aureus* (Cowans I) protein was coated with varying dilutions of anti *P. fluorescens* rabbit immunoglobulins to see the visible reaction as clumps. A co-agglutination reagent was prepared using both anti whole-cell sera and immunoglobulins. It was found that 1:2 dilution of anti *P. fluorescens* rabbit serum and 1:4 dilution of anti *P. fluorescens* rabbit immunoglobulins showed optimum agglutination in the co-agglutination test. Shelf life of co-agglutination reagent was ascertained and was found to be stable for three months (maximum number of months of study) even at room temperature. The result indicated that the co-agglutination test was fast, sensitive, inexpensive and can be used as an onsite immunodiagnostic test for the detection of *P. fluorescens*.

## Introduction

*Pseudomonas* species is frequently associated with fish diseases and is found on the eggs, skin, gills and intestine. Bacteria that are pathogenic to fish are mesophilic and usually require an optimum temperature of 10 to 30°C (Plumb 1994). *P. fluorescens* is a dominant component of freshwater ecosystem (Allen et.al. 1983).

*P. fluorescens* has been associated with septicemic and ulcerative condition in a wide range of fish species. *Pseudomonas* septicemia is a

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hemorrhagic condition of fish usually associated with stress or improper health management.

Proper health management plays an important role in a successful aquaculture practice. To achieve this, proper disease diagnosis and control measures should be provided. At present, these are being provided in sophisticated laboratories having trained manpower and upto date centralized automatic testing system. However, developing countries do not possess this kind of technology yet. Furthermore, decentralization of the diagnostic activities is necessary to avoid delay especially in places where fast communication is not available. *Clarias batrachus* is a highly priced fish. Its culture has not yet been intensified due to the paucity of hatchery raised seeds and serious disease problems associated with *Pseudomonas* and *Aeromonas* infection. Once an outbreak begins in the wild or in pond raised fish, it is difficult to control, as rapid growth of bacterium may cause irreparable systematic damage that leads to death. In response to this requirement, several fast and simple to operate on-site, non-laboratory tests have been developed. Dipstick and immunoconcentration assays were evolved from the classical ELISA in an attempt to simplify the procedure and to increase sensitivity for the disease diagnosis. However, these techniques are intended for individual testing. The main disadvantages are the lack in terms of quantity and the need for accurate dispensation of the reagents. On the other hand, an on-site, non-laboratory test such as the co-agglutination test requires only a small amount of reagent, economical, fast and sensitive. It does not require any instrumentation. Hence, efforts have been made to develop a co-agglutination test for the diagnosis of *P. fluorescens* infection.

## Materials and Methods

### *Bacterial strains and culture conditions*

The strain of bacterium *P. fluorescens* (MTCC103) was obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The strain of bacterium *S. aureus* (Cowans I) was obtained from the Fish Pathology Laboratory, Mumbai. Strains were identified according to Bergey's Manual of Systemic Bacteriology Vol. I (1991).

### *Immunization of rabbit, collection and purification of anti P. fluorescens rabbit serum*

Anti *P. fluorescens* antibodies were raised in rabbit using *P. fluorescens* as antigen. An 18-hour culture of *P. fluorescens* containing  $1.61 \times 10^{11}$  organisms·ml<sup>-1</sup> was used for the inoculation. The schedule followed (Malik 1992) for the immunization is given.

| Day      | Dose    | Route       |
|----------|---------|-------------|
| 1st day  | 0.1 cc  | Intravenous |
| 3rd day  | 0.25 cc | Intravenous |
| 5th day  | 0.5 cc  | Intravenous |
| 7th day  | 1 cc    | Intravenous |
| 9th day  | 2 cc    | Intravenous |
| 12th day | 3 cc    | Intravenous |

Seven days after the last injection, blood was collected from the marginal ear vein and subsequently serum was collected centrifuging the tubes at 2000 x g for 15 minutes. The titration of antiserum was performed by tube agglutination method as per the procedure of Schaperclaus (1954).

Immunoglobulins were separated from anti *P. fluorescens* rabbit serum by salt fractionation using ammonium sulphate precipitation method (Hudson and Hay 1991). For precipitation, 5 ml of serum was taken in a beaker and an equal volume of saturated ammonium sulphate solution at pH 8.0 was added by drops. The suspension was mixed for 30 minutes with constant stirring and subsequently centrifuged at 8000 x g for 30 minutes. The supernatant was discarded and the pellet was dissolved in 5 ml of normal saline (0.85% sodium chloride in distilled water) and precipitated again by gradual addition of equal volumes of saturated ammonium sulphate solution. The suspension was centrifuged and the cycle was repeated twice. The final precipitate was dissolved in 5 ml of saline. The suspension was dialyzed in a dialysis membrane (Sigma, USA) against three changes of PBS (sodium chloride 8.0 g, disodium hydrogen phosphate 1.44 g, disodium potassium phosphate 0.2 g, potassium chloride 0.2 g, distilled water 1000 ml, pH 7.2) over 36 to 48 hours to remove the salt. After dialysis the concentration of immunoglobulins was calculated.

### **Preparation of protein A**

Five ml of nutrient broth was inoculated with *S. aureus* (Cowans I) strain. After incubation at 37°C for 24 hours *S. aureus* was spread over Brain Heart Infusion agar medium (HiMedia, India) (calf brain infusion 20.0 g, beef heart infusion 25.0 g, peptone 1.0 g, sodium chloride 0.5 g, disodium hydrogen phosphate 0.25 g, dextrose 0.2 g, distilled water 100 ml, pH 7.4) in large sterile petri plates. These petri plates were incubated at 37°C for 48 hours.

The bacteria from petri plates was scrapped, collected, pooled and centrifuged at 1500 x g for 15 minutes. Pellets were treated with 2% formalin and left overnight at room temperature. Formalin treated cells were kept at 80°C for 15 minutes and immediately cooled in the ice bath. The cells were washed five times with PBS, pH 7.2 and required concentration of *S. aureus* suspension were prepared for the co-agglutination test.

### **Co-agglutination test procedure**

The co-agglutination test was performed as described by Collee et al. (1989). Protein A suspension was centrifuged, the supernatants were discarded and a 10% suspension was made in PBS. To one ml of 10% protein A suspension an equal volume of anti *P. fluorescens* serum was added. To another set, an equal volume of anti *P. fluorescens* rabbit immunoglobulins was added. After two hours incubation at room temperature the suspension was centrifuged at 1500 x g for 15 minutes and the supernatant was discarded. The pellet was suspended in an equal amount of PBS. This was used as the co-agglutination reagent. Agglutination was performed on a microscopic glass

slide by adding 50  $\mu$ l of sensitized *S. aureus* protein A (Cowans I) suspension to 50  $\mu$ l of broth culture of *P. fluorescens* ( $1 \times 10^6$  cfu·ml) and it was mixed using a toothpick. The visible agglutination reactions were recorded as positive. Suitable negative controls were included. Different volumes of reagent and antigen starting from 5  $\mu$ l each were tried for visible agglutination.

### **Artificial infection of *C. bacrachus* with *P. fluorescens* and co-agglutination test in infected fishes**

*C. bacrachus* weighing about 125 to 150 g were infected with 12 hours broth culture of *P. fluorescens* containing  $1.2 \times 10^9$  organisms per ml, 0.5 ml of broth culture was inoculated intraperitoneally and observed for visible signs and symptoms.

Infected fish were dissected and loopful (20 ml) of scrapping/fluids from different organs such as stomach, intestine, liver, spleen, kidney and skin separately on glass slide and 20 ml of co-agglutination reagent were added. They were mixed and observed for agglutination reaction. Clinically healthy fish were also tested.

### **Shelf life of co-agglutination reagent**

The reagent was stored at 37°C, room temperature and 4°C and tested for the reaction periodically; 150 ml aliquots of the reagent were taken in Eppendorf tubes. They were stored at required temperature and used as and when required.

## **Results and Discussion**

The present study hopes to make available a standardized immunoassay kit like a co-agglutination kit for monitoring *P. fluorescens* infection. Such kits are expected to provide assurance that the reagents and procedures are comparable between laboratories. Accordingly, the co-agglutination test was standardized and the shelf life of the reagents used in the diagnosis of the *P. fluorescens* infection was evaluated. The result of the co-agglutination test indicated that with these reagents, it is very easy to detect *P. fluorescens* infection in fishes.

The co-agglutination test developed by Kronvall (1973) was further standardized and the agglutination reaction was visible within two minutes, whereas Kronvall observed the agglutination reaction within 10 minutes. Co-agglutination has also been used for the detection of infectious pancreatic necrosis virus in the tissue samples of Atlantic Salmon by Taksdal and Thorud (1999). The bacterial meningitis in farmed rainbow trout has been diagnosed using agglutination test (Robert and Horne 1987).

The agglutination test developed by Toranzo et al. (1987) was used for detecting fish pathogens using whole cell antisera. In the present study, the co-agglutination test was performed using both antisera and immunoglobu-

lins. The titre of anti *P. fluorescens* rabbit serum was found to be 1:320. The results show that the immunoglobulins were sensitive and reactions were faster than that with the antisera. The result of the co-agglutination reaction is presented in figure 1. It was observed that 1:2 dilution of anti *P. fluorescens* rabbit serum and 1:4 dilution of anti *P. fluorescens* rabbit immunoglobulins gave optimum agglutination. The results are presented in tables 1 and 2. The optimum concentration for good visible reaction was found to be 50  $\mu$ l of the co-agglutination reagent and 50  $\mu$ l of the antigen. The result is presented in figure 3. The specificity of the co-agglutination reagent was confirmed by the absence of cross reactivity using the co-agglutination reagent with *Edwardsiella tarda*, *Vibrio alginolyticus*, *Aeromonas hydrophila*, and was in confirmation with the co-agglutination test developed by Lalitha (1991).

In the present study, it has been found that compared to conventional slide agglutination, the co-agglutination presents more advantages being simple, sensitive and economical. The co-agglutination test was carried out

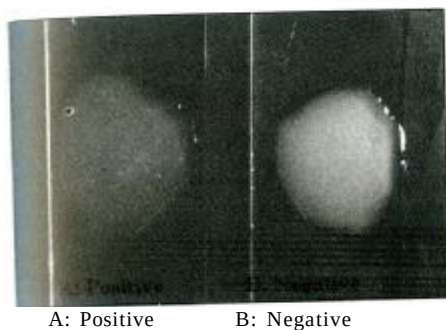


Fig. 1. Co-agglutination test with *P. fluorescens*

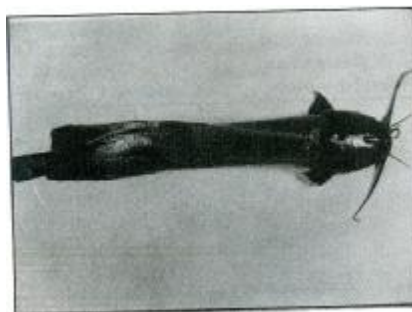


Fig. 2. *C. batrachus* showing signs of *P. fluorescens* infection

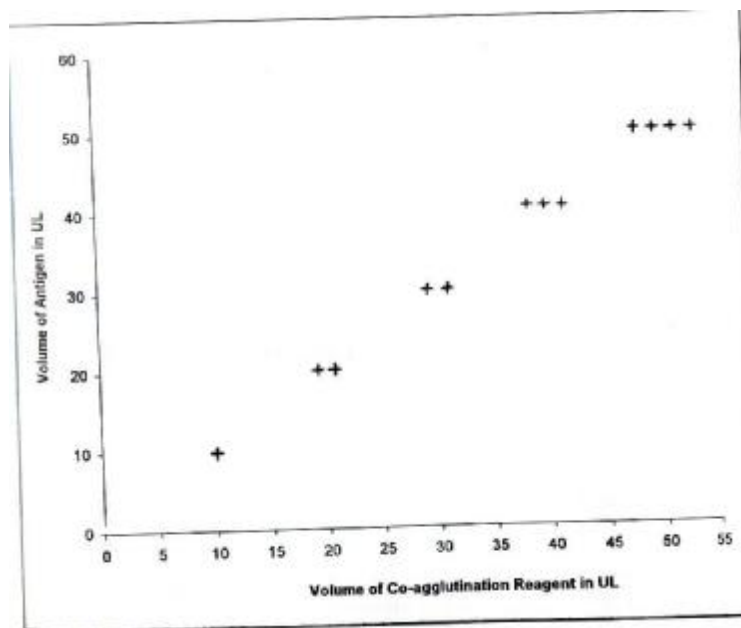


Fig. 3. Optimization of volume of co-agglutination reagents.

using both *P. fluorescens* and inoculum from different organs of *C. batrachus* infected with *P. fluorescens*. In both cases reaction was fast and specific. However, agglutination reaction was found to be slow with inoculum from the liver and spleen. For artificial infection of the fishes, 0.5 ml<sup>-1</sup> of 12-hour broth culture containing 1.2 x 10<sup>7</sup> number of organism·ml<sup>-1</sup> was injected. Sloughing of skin and hemorrhages on the surfaces, disintegration of pectoral and dorsal fins and sluggishness were the prominent external signs of the infected fishes.

The stability of the reagent used in the co-agglutination kit is important for a reproducible performance of the kit. The kit is likely to be exposed to various temperatures due to lack of maintenance of cold chain during transportation to distant places. The kit is also likely to be stored in higher temperature due to lack of refrigerators. Hence, in the present study attempts were made to study the shelf life of the co-agglutination reagent

Table 1. Optimum concentration of anti *P. flourescens* rabbit serum for sensitizing protein A

| Dilution of anti<br><i>P. fluorescens</i><br>rabbit serum | Test<br>Co-agglutination reagent<br>(Antisera + Protein A) + <i>P. flourescens</i> | Control<br>Co-agglutination reagent +<br><i>V. alginolyticus</i> |
|-----------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------|
| 1:2                                                       | +                                                                                  | -                                                                |
| 1:4                                                       | -                                                                                  | -                                                                |
| 1:8                                                       | -                                                                                  | -                                                                |
| 1:16                                                      | -                                                                                  | -                                                                |
| 1:32                                                      | -                                                                                  | -                                                                |
| 1:64                                                      | -                                                                                  | -                                                                |
| 1:128                                                     | -                                                                                  | -                                                                |

Table 2. Optimum concentration of anti *P. flourescens* rabbit immunoglobulins for sensitizing protein A

| Dilution of anti<br><i>P. fluorescens</i><br>rabbit serum | Test<br>Co-agglutination reagent<br>(Antisera + Protein A) + <i>P. flourescens</i> | Control<br>Co-agglutination reagent +<br><i>V. alginolyticus</i> |
|-----------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------|
| 1:2                                                       | +                                                                                  | -                                                                |
| 1:4                                                       | +                                                                                  | -                                                                |
| 1:8                                                       | -                                                                                  | -                                                                |
| 1:16                                                      | -                                                                                  | -                                                                |
| 1:32                                                      | -                                                                                  | -                                                                |
| 1:64                                                      | -                                                                                  | -                                                                |
| 1:128                                                     | -                                                                                  | -                                                                |

Table 3. Co-agglutination test in artificially infected fish (*C. batrachus*)

| S. no. | Inoculum from organs         | Reaction |
|--------|------------------------------|----------|
| 1      | Liver                        | +++      |
| 2      | Kidney                       | ++++     |
| 3      | Stomach                      | ++++     |
| 4      | Ulcer                        | ++++     |
| 5      | Spleen                       | ++       |
| 6      | Skin scrapping               | ++++     |
| 7      | Inoculum from normal tissues | -        |

at various storage temperatures viz. 37°C, room temperature (25 to 30 °C) and at 4 °C. The result of the study reveals that the co-agglutination reagent (immunoglobulin + Protein A) did not lose its stability even when stored at room temperature for three months (maximum number of months of study). Whereas reagents stored at 37°C reduced their reactivity (Table 4). In the same way, the stability of the co-agglutination reagent (antisera + protein A) was found to be stable only at 4°C (Table 5).

In conclusion, the present study paved the way for an understanding of the co-agglutination reaction and development of each to determine disease incidence. The onsite, nonlaboratory test was standardized and can be

Table 4. Shelf life of co-agglutination reagent protein A + anti *P. fluorescens* rabbit immunoglobulins)

| Period in weeks | Immunoglobulins + protein A storage temperature |      |      |
|-----------------|-------------------------------------------------|------|------|
|                 | 37 °C                                           | RT   | 4 °C |
| 1               | ++++*                                           | ++++ | ++++ |
| 2               | ++++                                            | ++++ | ++++ |
| 3               | ++++                                            | ++++ | ++++ |
| 4               | ++++                                            | ++++ | ++++ |
| 5               | ++++                                            | ++++ | ++++ |
| 6               | ++++                                            | ++++ | ++++ |
| 7               | ++++                                            | ++++ | ++++ |
| 8               | ++++                                            | ++++ | ++++ |
| 9               | ++++                                            | ++++ | ++++ |
| 10              | ++++                                            | ++++ | ++++ |
| 11              | ++++                                            | ++++ | ++++ |
| 12              | +++                                             | ++++ | ++++ |
| 13              | ++                                              | ++++ | ++++ |

RT indicates room temperature (25 to 30°C)

\* The reactions are scored 4+ on formation of distinct visible clumps.

Table 5. Shelf life of co-agglutination reagent protein A + anti *P. fluorescens* rabbit serum

| Period in weeks | Immunoglobulins + protein A storage temperature |      |      |
|-----------------|-------------------------------------------------|------|------|
|                 | 37 °C                                           | RT   | 4 °C |
| 1               | ++++*                                           | ++++ | ++++ |
| 2               | ++++                                            | ++++ | ++++ |
| 3               | ++++                                            | ++++ | ++++ |
| 4               | ++++                                            | ++++ | ++++ |
| 5               | ++++                                            | ++++ | ++++ |
| 6               | ++++                                            | ++++ | ++++ |
| 7               | ++++                                            | ++++ | ++++ |
| 8               | ++++                                            | ++++ | ++++ |
| 9               | ++++                                            | ++++ | ++++ |
| 10              | ++++                                            | ++++ | ++++ |
| 11              | ++++                                            | ++++ | ++++ |
| 12              | +++                                             | ++++ | ++++ |
| 13              | ++                                              | ++++ | ++++ |

RT indicates room temperature (25 to 30°C)

\*The reactions are scored 4+ on formation of distinct visible clumps.

used in the field. The test is simple and sensitive. Though the results suggest that it can be used in the detection of *P. fluorescens* infection, further studies are still required to enhance shelf life.

### Acknowledgment

The authors wish to acknowledge the Director, Central Institute of Fisheries Education, Mumbai for providing the facilities to carry out this research work.

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