Elisa preliminary studies of immobilization and specific detection of bacterial strains

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Abstract

The paper aims to emphasize the specific detection of bacterial strains using enzyme-linked immunosorbent assay. The assay is based on the specific binding of polyclonal antibody anti-E. coli tagged with FITC to E.coli and monoclonal antibody anti-Ps. aeruginosa tagged with Alexa Fluor 647 tagged to Ps. aeruginosa and on subsequent enzymatic immunological demonstration of the conjugated enzyme. In this experiment, the negative control was the Salmonella enterica strain. The two antibodies had no interaction with the negative control, instead, they were specific for E. coli and Ps. aeruginosa strains. When both strains were in the same well, the fluorescence intensity given by the presence of E. coli was 2.3 times higher than that given by Ps. aeruginosa, and the intensity of fluorescence decreased if there are both bacterial strains in the wells.

Keywords: *antigen-antibody, pathogen bacteria, enzyme-linked immunosorbent assay*

INTRODUCTION

The emergence of pollutants and pathogens, mainly antimicrobial-resistant bacteria, in the environment has been an issue in the last decades and continues to be a critical threat worldwide both for the aquatic systems and human health.

Nosocomial infections or in-hospital infections affect a large number of patients globally, representing 7% in developed countries and 10% in developing countries. Nosocomial pathogens can be bacteria, viruses, and fungus. WHO estimates that approximately 15% of hospitalized patients suffer from this type of infection [1]. More than 51% of intensive care patients are affected by in-hospital infections, according to the Extended Prevalence of Infection in Intensive Care (EPIC II) study [2].

Some extensive studies in Europe and the USA show that nosocomial infections incidence density ranged from 13,0 to 20,3 episodes per thousand patient-days [3].

The most implicated pathogens in nosocomial infections are *E. coli* and *Pseudomonas aeruginosa*. *Escherichia coli* is a Gram-negative bacillus present in the human and animal intestines and in the feces, which led to this microorganism being considered an indicator of fecal pollution environment, especially for water bodies [4].

Pseudomonas aeruginosa is also a Gram-negative, commensal bacillus of the digestive tract. It is increasingly isolated from the hospital environment on various surfaces or even from instruments, medical devices, antiseptic solutions, or drugs [4].

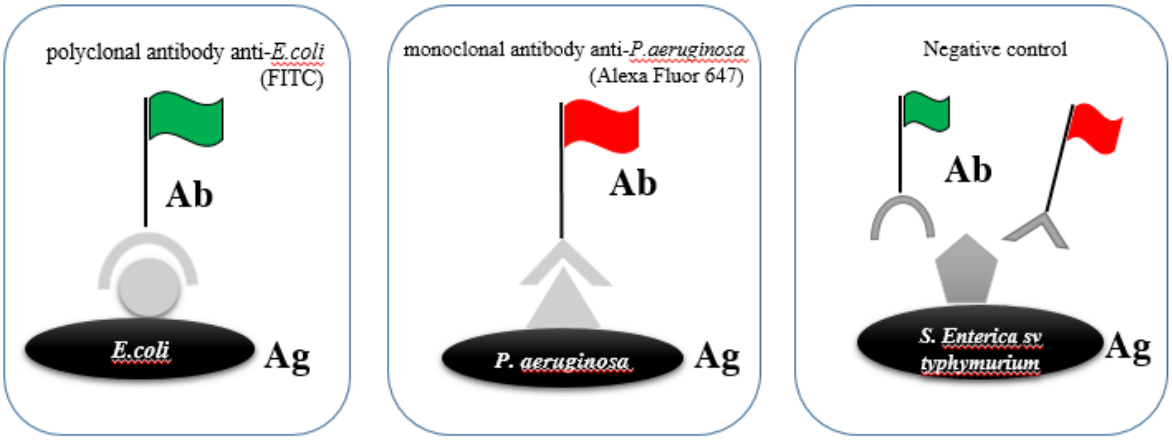
Water-borne pathogen contamination of water resources and related infectious diseases has been a major water quality concern throughout the world [5-7].

Accelerated pathogens spreading over large areas require fast and specific detection methods for early and successful management of antimicrobial resistant bacteria [8]. Conventional methods of pathogen detection are usually time-consuming, expensive, and often insensitive [9]. These are generally based on metabolic reactions of bacteria, using different types (nutritive and specific) of culture media and requiring more serial replications. One of these methods to assess water body quality has been the use of fecal indicator bacteria (e.g. *Escherichia coli*) to suggest the presence of harmful fecal pollution [10].

At the present, there is a tendency to switch the conventional detection methods to more advanced molecular methods mainly based on PCR-based methods, next-generation sequencing techniques, and the enzyme-linked immunosorbent assays (ELISA).

This immunological technique is highly sensitive, time-effective, and highly specific due to its antigen antibody-like interaction

specificity [11, 12].
The ELISA technique has been successfully applied in various research fields such as clinical diagnosis, quality control, and pathological studies [13-18].
All microbial species have unique antigens that could be used as specific targets for quantitative detection in ELISA experimental settings [19].
The goal of this study was to use the Elisa method to detect and quantify a specific antigen in a sample with a specific antibody conjugated to an enzyme. The antigen is identified by the enzyme-conjugated antibody, after incubation, the appearance of the color indicates the presence of the antigen, and the intensity of the color denotes the amount of antigen. The basic concept of antigen-antibody interaction is presented in Figure 1 [20].



(a) (b) (c)

Fig. 1. a) Polyclonal anti-*E.coli* antibody recognizes only one specific *E.coli* marker; b) the anti-*Ps.aeruginosa* monoclonal antibody recognizes only one specific marker *Ps. aeruginosa;* c) neither of the two antibodies has a specific interaction with *S.enterica sv. Typhymurium* (this strain representing the negative control)

In this paper, the ELISA assay was used as an alternative to the conventional methods of pathogen detection from the aquatic environment. The rapidity, specificity and cost-effectiveness of ELISA proved to be a step forward for bacterial detection compared to the classical and laborious bacterial identification method, based on repeated bacterial growth on selective medium. A fast and reliable result is very essential in designing a fast response for the management of a health-threatening situation. Two Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* with a pathogenic potential were specifically detected by the fluorescence intensity method, using *Salmonella enterica* as a negative control.

EXPERIMENTAL PART

Elisa tests use two types of synthetic substances: the conjugate (the antibody conjugated to an enzyme) and the substrate (the enzyme-substrate in the conjugate). These tests are performed on nitrocellulose as solid support. The sample containing the test antigen is added to the solid support (in the wells of the plates) then the conjugate is added. If the sample contains the target antigen, the specific antibodies in the conjugate will bind to it, otherwise, these bonds will not form, and by washing the conjugate will be removed. The enzyme-substrate contained in the conjugate is then added. If the conjugate has remained bound by the antigen-antibody reaction, the enzyme-substrate reaction will take place as a color reaction. The appearance of color demonstrates the presence of the complex 'target antigen + enzyme-conjugated antibody + enzyme-substrate' [21].

Bacterial growth

Three Gram-negative bacterial strains *Escherichia coli* (ATCC 25922 PK/5), *Pseudomonas aeruginosa* (ATCC 27853 PK/5), and *Salmonella enterica sv. Typhymurium*

(ATCC 14028 PK/5) purchased from ATCC (International Center for the Authentication, Storage and Production of Microorganisms and Cell Lines) were grown on tryptone soya agar (Oxoid, UK) and incubated overnight (O/N) at 37°C. Then one colony was transferred in 10 ml Lauryl Sulphate Broth (Oxoid, UK) in a shaking incubator (New Brunswick Scientific, Innova 44) at 37°C for 24h. Bacterial growth was monitored by measuring the absorbance at 600 nm using a UV-VIS spectrometer (VWR International, USA).

Chemical reagents

Bovine serum albumin 10% (BSA) (Sigma-Aldrich, USA) was used as a blocking solution and Phosphate Buffered Saline (PBS 1x) (Sigma-Aldrich, USA) with 0.5% Tween 20 Solution (Bio-Rad Laboratories, USA) were used as a washing solution. The chemical reagents were purchased from Redox Lab Supplies SRL and Dialab Solution SRL (Romania).

ELISA assay

96-well microplate was incubated 2h at room temperature with 100 µl of 0.4 OD_{600nm} bacterial suspensions of *Escherichia coli*, *Pseudomonas aeruginosa* or *Salmonella enterica sv. Typhymurium.* After incubation time, the plate was washed three times with 100 µl 0.5% Tween 20 in PBS, then the plate was incubated with 100 µl 10% BSA for 1h at room temperature, followed by washing three times with 100 µl 0.5% Tween 20 in PBS. A 100 µl antibody solution (dilution of 1:100 in PBS 1x) was incubated 30' at room temperature; polyclonal anti-*Escherichia coli* tagged with FITC (Novus Biologicals, CO, USA) and monoclonal anti-*Pseudomonas aeruginosa* tagged with Alexa Fluor 647 (Novus Biologicals, CO, USA). After antibody incubation time and 0.5% Tween 20 in PBS washing step, the specific bacterial detection was carried out at a Clariostar Microplate reader (BMG Labtech, Germany) based on the fluorescence characteristics (Table 1). The bacterial strain *Salmonella enterica sv. thyphymurium* was used as a negative control.

Table 1. Fluorescence characteristic parameters

Fluorophores type	Wavelength settings (nm)			Gain (0...4095)		
	Excitation	Dichroic	Emission	I	II	III
Fluorescein isothiocyanate (FITC)	483-14	502.5	530-30	800	1500	2000
AlexaFluor 647	625-30	652.5	680-30	1400	2000	2500

RESULTS AND DISCUSSIONS

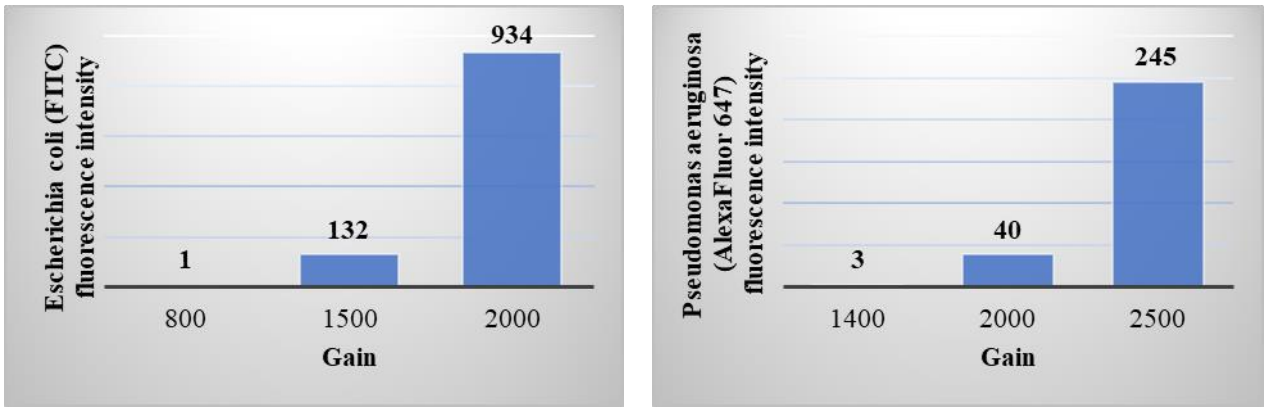
Specific bacterial Gram-negative strains with fluorescently tagged antibody were detected by enzyme-linked immunosorbent assay. One colony from each bacterial strain isolated from the solid growth medium was incubated in specific liquid media and all three bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica sv. thyphymurium*) showed a robust growth after about 4 hours of incubation at 37°C reaching a high optical density up to 1.5 OD measured at 600 nm (Table 2).

Table 2. The optical density of bacterial strains at 600 nm

Bacterial strains	Optical density (OD) measured at 600 nm
Escherichia coli	1.477
Pseudomonas aeruginosa	0.883
Salmonella enterica sv. thyphymurium	0.906

Establishing the proper gain based on the fluorescence characteristics

The antibodies labeled with FITC and AlexaFluor 647 were tested to establish an optimal intensity read. The tested gains were analyzed at 800, 1500, and 2000 for FITC as well as 1400, 2000, and 2500 for AlexaFluor 647. The best response was given by the gain 2000 for *Escherichia coli* strain- polyclonal antibody anti-*E. coli* tagged with FITC and gain 2500 for *Pseudomonas aeruginosa* monoclonal antibody anti-*Ps. aeruginosa* tagged with AlexaFluor 647 (Figure 2).



(a) (b)

Fig. 2. The influence of gain on the fluorescence intensity: a) *E. coli* with FITC, b) *Ps. aeruginosa* with Alexa Fluor 647

Bacterial detection with specific antibodies labeled with fluorochromes

A bacterial density of 0.4 OD_{600nm} was incubated in the presence or absence of a polyclonal anti-*Escherichia coli* tagged with FITC and monoclonal antibody anti-*Ps. aeruginosa* tagged with Alexa Fluor 647 as presented in the experiment template (Table 3).

Table 3. Enzyme-linked immunosorbent assay template

Control	*Escherichia coli*	*Pseudomonas aeruginosa*	*Salmonella enterica sv. Typhymurium*	*Escherichia coli* + *Pseudomonas aeruginosa*
PBS 1x	FITC	AlexaFluor 647	FITC+AlexaFluor 647	FITC+ AlexaFluor 647
PBS 1x	FITC	AlexaFluor 647	FITC+AlexaFluor 647	FITC+ AlexaFluor 647

The controls PBS and *Salmonella enterica sv. Typhymurium* showed no interaction with the antibodies. On the other hand, the antibodies specificity was showed. *Escherichia coli* incubated in presence of polyclonal anti-*Escherichia coli* tagged with FITC had a specific signal of 934 at a gain of 2000 (Fig. 3-blue), respective *Pseudomonas aeruginosa* in presence of monoclonal anti-*Pseudomonas aeruginosa* tagged with Alexa Fluor 647 had a specific signal of 245 at a gain 2500 (Fig. 3-green).

The signal given by the fluorescence of the *E .coli*-FITC system was about 4 times more intense than that given by *Ps. aeruginosa*-Alexa Fluor 647, for the situation in which there was only one bacterial strain in the well.

If there was only a bacterial strain in the wells compared to the wells in which both strains were, the fluorescence intensity decreased by approx. 40 % in the case of *E. coli*, respectively approx. 50 % in the case of *Ps. aeruginosa*.

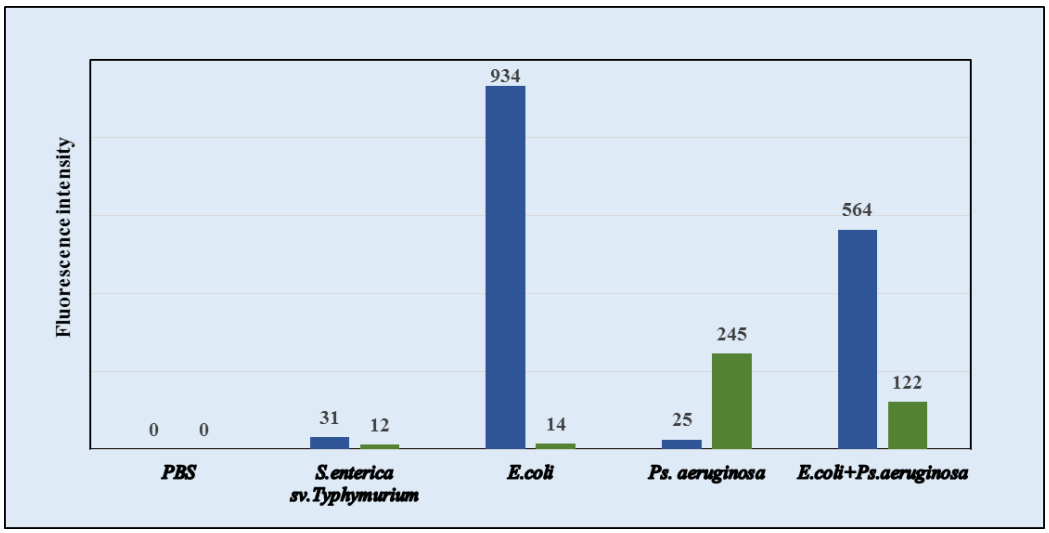


Fig. 3. Specific detection of *Escherichia coli* and *Pseudomonas aeruginosa* with antibodies labeled with FITC or AlexaFluor 647 fluorochromes

CONCLUSIONS

Specific and rapid detection of Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* from cultures with reference strains was performed by direct enzyme-linked immunosorbent assay in presence of polyclonal anti-*Escherichia coli* tagged with FITC and monoclonal antibody anti-*Pseudomonas aeruginosa* tagged with Alexa Fluor 647. The results emphasized a specific detection for both analyzed strains.

In the case of *Escherichia coli*, the intensity of fluorescence was 3.8 times higher than for *P. aeruginosa* (in the situation when there was only one bacterial strain in the wells).

When both strains were in the same well, the fluorescence intensity given by the presence of *E. coli* was 2.3 times higher than that given by *Ps. aeruginosa*.

The intensity of fluorescence decreased if there were both bacterial strains in the wells compared to the situation where there was only one in the well, with approx. 40 % in the case of *E.coli*, respectively approx. 50 % in the case of *Ps. aeruginosa*.

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