ASSAYS FOR DETECTION OF *PSEUDOMONAS AERUGINOSA* WITH SPECIAL REFERENCE TO PHAGE BASED BIOSENSORS

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ABSTRACT

Water safety is a global health goal and water borne diseases take a major crisis on health. Therefore, detection of microbial pathogens in water timely and accurately is of utmost importance. *Pseudomonas aeruginosa*, a bacterium included in the list of water borne pathogens by WHO in their "Guidelines for Drinking Water Quality", causes serious implications in animals as well as humans especially in children and immune-compromised patients. This review discusses the various methods like culture methods, immnunoassays, molecular assays, flurometric assays and biosensors for detection of *P. aeruginosa* with special reference to bacteriophage based biosensors. Phages are the natural enemies of bacteria which specifically recognize and infect a particular species of bacteria. Now bacteriophages are being employed as the bio-recognition element of a biosensor as they are highly specific and applicable for all the bacterial species including *P. aeruginosa*. The use of phage based biosensor could lead to a revolution in the field of disease diagnosis, detection of food and water contaminants and environmental pollution. This would be of critical importance to microbiologists, clinicians, public health personnel and policy makers.

Key words: *Pseudomonas aeruginosa*, Phage, Biosensor, Water contaminant, Molecular Diagnosis

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Introduction

There has been a remarkable progress in prevention, control and even eradication of infectious diseases with improved hygiene and development of antimicrobials and vaccines. However, bacterial diseases still remain a leading cause of global disease burden with high morbidity and mortality especially in the developing world. According to World Health Organization, every year more than 3.4 million people die as a result of water-borne diseases, making it the leading cause of disease and death around the world [42]. One of the most dominant causative agents of these water borne diseases is *Pseudomonas aeruginosa*, a bacteria included in the list of water-borne pathogens by WHO in their "Guidelines for Drinking Water Quality" [43]. This bacterium has been found to cause serious implications especially in children and immune-compromised patients besides causing general bacteria associated infections. Their control requires continuing surveillance, research and training, better diagnostic facilities and improved public health system [9].

The conventional methods for the detection of bacteria require several days to give results because they rely on the ability of the organisms to multiply to visible colonies. Various methods which have been developed for the detection of different infectious agents like *P. aeruginosa* includes gram-staining analysis, incubation and biochemistry reaction identification, blood serum identification, quartz crystal microbalance (QCM) techniques, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), immunoassay and many more [34]. But it has been found that the available methods suffer from one or the other limitations which calls for a new and improved methodology for the rapid detection and quantification of this bacterium. Biosensor is the upcoming technology of recent times as it is rapid, more sensitive, more specific and cost effective compared to most of the other conventional approaches used in bacteria identification and enumeration.

Bacteriophages are the natural enemies of bacteria which specifically recognize and infect a particular species of bacteria. They were employed in earlier times to cure bacterial infections but the discovery of antibiotics prevented further advances and improvements in this field. However, the development of antibiotic-resistance among various bacterial species renewed the interest of scientists around the world in these tiny creatures. Meanwhile, bacteriophages are now being employed as the bio-recognition element of a biosensor as they are

highly specific and applicable for all the bacterial species. The use of phage based biosensor could lead to a revolution in the field of disease diagnosis, detection of food and water contaminants and environmental pollution.

Pseudomonas aeruginosa as pathogen

Pseudomonas aeruginosa is a common bacterium which causes disease in animals including humans [12]. *P. aeruginosa* is a bacterium with ubiquitous occurrence in the environment [17,21]; in soil; potable water sources like tap water, wells, and sinks as well as other water sources like streams, lakes, hot tubs and rivers; skin flora of animals; even in respiratory therapy equipment, catheters, dialysis tubing and most man-made environments [24,39]. It can survive in water at 37°C for up to 300 days [4]. It is a gram-negative, non-sporulating, motile, rod shaped bacterium. The bacterium is capable of both aerobic and anaerobic growth. The enumeration of *P. aeruginosa* has been made mandatory by some regulatory authorities when testing mineral waters, for example: Natural Mineral Waters Regulations [40]. As water is a critical raw material used in the manufacture of pharmaceutical and cosmetic products, hence, presence of *P. aeruginosa* contamination is not only a concern in case of potable water but also in water meant for industrial and medical purposes.

The literature cited here supports the importance of rapid detection of *P. aeruginosa* in water, as a potential source of infection. The coliform group of bacteria, and to a lesser degree the fecal streptococci, have been used more frequently than other groups of organisms as indicators of microbial contamination in water. However, the use of the coliform test as the sole indicator system is debatable and has been questioned. Moreover, it has been reported by several authors that conventional method of relating the fecal coliform count with the presence or absence of a pathogenic bacteria is not much successful in case of *P. aeruginosa*. Some people from the scientific community feel that the use of *P. aeruginosa* should also be considered as an index of microbial contamination of water since this organism has been isolated from both potable and insufficiently treated water supplies, even in the absence of fecal coliforms [18].

Methods for detection of _P. aeruginosa_

Various methods have been developed for the detection of *P. aeruginosa* including gram-staining analysis, incubation and biochemistry reaction identification, blood serum identification, QCM techniques, ELISA, PCR, immunoassays and many more [34].

Culture Methods

Several selective media have been developed for the isolation and cultivation of *P. aeruginosa* [15,5,37,23,44,6]. A detailed description of the various isolation media like skim milk agar, cetrimide (Pseudosel), Flo agar, F agar, P agar, Warburton media, and MacConkey agar for *P. aeruginosa* has earlier been documented [17]. Gram-staining analysis cannot distinguish *P. aeruginosa* from other gram-negative Bacillus; it merely gives a primary estimation [34]. The culture methods do not detect dead bacteria, which is an advantage but however, these cultivation based methods require days from initiation to read-out, also, interpretation of results may become difficult because of interfering microflora [14,31]. Also, viable but not cultivable bacteria, that could be potentially pathogenic, may not be detected in this way [24,8]. Moreover, the identification of certain types of pathogens by culturing often results in false negatives due to a high background level of competing microorganisms [46].

Immunoassays

Immunoassays for the detection of *P. aeruginosa* have also been described. For instance, a double antibody sandwich ELISA was designed to target the lipopolysaccharides (LPS) from eight serotypes of *P. aeruginosa* (O1, O2, 5, 16, O3, O4, O6, O9, O10, and O11) [26]. Another study was conducted to develop an Immunofluorescent-Antibody Test for rapid identification of *P. aeruginosa* in blood cultures. They reported that bright uniform immunofluorescence signal because of conjugation of monoclonal antibody with fluorescein isothiocyanate was observed when each of the 17 international serotypes as well as 14 additional isolates of *P. aeruginosa* was examined. No immunofluorescent staining was observed when 37 other gram-negative and 15 gram-positive species were studied [10]. Based on the observation that clinical isolates of *P. aeruginosa* express a specialized protein secretion system, called the type III secretion system (T3SS), a study was conducted to design an indirect ELISA for quantitative assessment of type III virulence phenotypes of *P. aeruginosa* isolates. It was reported that the results of this assay were concordant with immunoblot detection of the secreted antigens for 73 of 74 isolates [29].

Molecular Assays

The first study involving the use of PCR method for environmental monitoring of *P. aeruginosa* was based on the specific amplification of exotoxin A (*ETA*) structural gene sequence (396 bp). The assay was found to be able to detect as few as 5 to 10 cells in a 10-ml water sample or 0.1 pg

of *P. aeruginosa* DNA per reaction mixture (5µl). Moreover, ten-times-lower concentrations were detected by hybridization with a digoxigenin-labeled oligonucleotide probe internal to the PCR product. This method was used to detect *P. aeruginosa* in animal cage water samples at a level of 40 cells per ml [24]. A study was initiated to design two PCR assays to provide genus- or species- level identification of *P. aeruginosa* using 16S rDNA sequence data. They reported that the assay showed 100% sensitivity and specificity [38].

A multiplex PCR test based on the simultaneous amplification of two lipoprotein genes, *oprI* and *oprL*, was designed and evaluated for its ability to directly detect fluorescent Pseudomonads (amplification of *oprI* open reading frame, 249 bp) and *P. aeruginosa* (amplification of *oprL* open reading frame, 504 bp) in clinical material. The lower detection level for *P. aeruginosa* was estimated to be 10^2 cells/ml. Preliminary evaluation on testing skin biopsy specimens from patients with burns (n = 14) and sputum samples from cystic fibrosis patients (n = 49) and other patients (n = 19) showed 100% sensitivity and 74% specificity in comparison with culture [41].

Among the molecular biology-based methods, DNA microarray technology presents the potential of direct and rapid identification of multiple DNA sequences. The use of multiplex-PCR as a co-adjuvant for DNA microarrays in pathogen detection was investigated to overcome the problem of low pathogen count in clinical and environmental samples [33]. This study designed a DNA microarray consisting of 930 gene segments from various relevant bacterial species including *P. aeruginosa*.

Due to the large number of pathogenic microorganisms, one of the most important safety and quality indices in the control of water areas and food products is the pathogen viability detection, as only the living cells are harmful [7]. However, molecular assays based on DNA detection fails to differentiate between dead and live bacteria and do not provide any insight about bacterial viability which becomes more important when analyzing the effect of a bactericidal agent or process [46].

Fluoremetric Assay

Hydrolytic enzymes like elastase and LasA protease produced by *P. aeruginosa* were targeted in a study to develop a fluoremetric assay for the continous monitoring of this bacterium. Hydrolysis of fluorogenic substrates was monitored by analytical RP-HPLC [13].

Diagnostic Kits

Various diagnostic kits have also been developed to help in rapid detection of *P. aeruginosa* like PrimerDesign^{TM} Ltd; Milliflex® (Millipore); TaqMan® (Applied Biosystems), AquaSafe, Sigma Aldrich, Potaflex® (Wagtech WTD), Technostart, etc.

Biosensors

Biosensors is the latest technology being developed today for rapid detection of micro-organisms in food, water, cosmetic, pharmaceutical and industrial products. Biosensors are also referred as immunosensors, biochips, biocomputers, glucometers, etc. The name "biosensor" itself signifies that the device is a combination of two parts: (i) a bio-element, and (ii) a sensor-element. A commonly cited definition is: "a biosensor is a chemical sensing device in which a biologically derived recognition entity is coupled to a transducer, to allow the quantitative development of some complex biochemical parameter". A specific "bio" element (say, enzyme) recognizes a specific analyte and the "sensor" element transduces the change in the biomolecule into an electrical signal. The "bio" element is very specific to the analyte to which it is sensitive. It does not recognize other analytes.

Biosensor can also be defined as an analytical device that couples microorganisms with a transducer to produce a signal proportional to the analyte concentration to enable rapid, accurate and sensitive detection of target analytes in fields as diverse as medicine, environmental monitoring, defense, food processing and safety. This signal can result from a change in protons concentration, release or uptake of gases, light emission, absorption and so forth, brought about by the metabolism of the target compound by the biological recognition element. The transducer converts this biological signal into a measurable response such as current, potential or absorption of light through electrochemical or optical means, which can be further amplified, processed and stored for later analysis [28]. Biomolecules such as enzymes, antibodies, receptors, organelles and microorganisms as well as animal and plant cells or tissues have been used as biological sensing elements. Biosensors can be of many types depending upon the variety of transducers used such as amperometric, potentiometric, calorimetric, conductimetric, colorimetric, luminescence, fluorescence, etc. Biosensor technology is the upcoming technology of recent times as it is rapid, more sensitive, more specific and cost effective compared to most of the other conventional approaches used in bacteria identification and enumeration.

Wireless Magneto-elastic Biosensors: A wireless magnetoelastic sensing device was fabricated for the real-time quantification of *P. aeruginosa* concentrations. The sensor was made by coating a magnetoelastic ribbon with a polyurethane protecting film. In response to an externally applied time varying magnetic field, the magnetoelastic sensor vibrates at a resonance frequency that can be remotely determined by monitoring the magnetic flux emitted by the sensor. The resonance frequency changes in response to property changes of a liquid culture medium and bacteria adhesion to the sensor as *P. aeruginosa* consumes nutrients from the culture medium in growth and reproduction. The effects of properties (conductivity, viscosity, mass) were investigated with quartz crystal microbalance (QCM), microscopy imaging, and conductivity measurement. Using the described technique the sensor was able to directly quantify *P. aeruginosa* concentrations of 10^3 to 10^8 cells/ml, with a detection limit of 10^3 cells/ml at a noise level of ~20 Hz. The lack of any physical connections between the sensor and the monitoring electronics facilitates aseptic operation, and makes the sensor platform ideally suited for monitoring bacteria from within, for example, sealed food containers [34]. As biofilm formation is one of the important characteristics of *P. aeruginosa*, a sensor was developed for the real-time evaluation of the biofilm formation process by this bacterium using a wireless, passive magnetoelastic-sensing device consisting of a polyurethane-coated magnetostrictive ribbon which is placed in a flowing system, and then both the resonance frequency and amplitude of the sensor are wirelessly monitored through magnetic field telemetry to monitor biofilm formation [35].

Immunosensor: A label-free immunosensor system for detecting *P. aeruginosa* was developed [25]. Four types of anti-*P. aeruginosa* antibody were individually chemisorbed onto one-side gold electrodes of piezoelectric quartz crystals according to a thiolated antibody coupling procedure initiated with a thiol-cleavable heterobifunctional cross-linker, sulfosuccinimidyl-6-[3-(2-pyridyldithio) propionamido] hexanoate. The frequency shifts obtained were quite specific according to the antibody types and *P. aeruginosa* strains. The biosensor responses to varying concentrations of the *P. aeruginosa* cells ranging from 1.3×10^7 to 1.3×10^8 CFU/ml were determined as 17–176 Hz and a linear calibration curve was obtained by plotting the responses in a double-logarithmic scale. The selectivity of the biosensor between *P. aeruginosa* and *Xanthomonas* spp., which both belong to the aerobic pseudomonads, was however, not so good

owing to the property of the antibody used. The sensor chip could be reused at least seven times without an appreciable decrease in sensitivity.

ATP Bioluminescence Assay: ATP is a key molecule in the metabolism of both mammalian and bacterial cells and can be used to quantify cell numbers. The amount of ATP present in a sample can serve as an indicator of the amount of cellular activity present in it. The ATP bioluminescent assay is a sensitive, simple and rapid method of accurately determining levels of microbial ATP and thus, the number of bacteria present in a sample [27]. An essential part of this assay is a cell lysis step that employs agents to release intracellular ATP. The released ATP is then measured using a bioluminescent reaction with firefly luciferase [45]. Firefly luciferase catalyzes the oxidation of luciferin while transforming the energy derived from ATP into light in the presence of Mg^{2+} and molecular oxygen. The bioluminescence color of firefly luciferases is identified by the luciferase structure and assay conditions [30].

Optimization and validation of ATP bioluminescence assay was done to detect the presence of microbial contaminants like *P. aeruginosa* in high fluoride and triclosan dentifrice formulation to ensure faster product release and quality evaluation [19]. Bioluminescence was also used as a sensitive marker for the detection of *Pseudomonas* species in the rhizosphere. The theoretical detection limit of this assay is about 10^3 bacterial cells, although in practice 10^4 - 10^5 cells is more typical [3]. To enhance the bioluminescence signals of the assay, investigations were carried out whether ADP glucose pyrophosphorylase (AGG Pase), which catalyzes the formation of ATP and glucose-1-phosphate from ADPglc and PPi (inorganic pyrophosphate), could increase the luminescence signals of luciferase-based assays. It was observed that the AGPPase/ADPglc-based ATP regeneration system not only showed much lower backgrounds than the ATP sulfurylase/APS system but also stabilized the signals of the conventional luciferase-based ATP measurement assays. The AGPPase based assay could be used to measure both PPi and ATP quantitatively and showed 1.5- to 4.0-fold slight increases in a 10-min assay. The method could also be used to stabilize the luminescence signals in detection of *Escherichia coli*, *P. aeruginosa*, and *Bacillus cereus* in either broth or biofilm [27].

Phage based biosensors: A bacteriophage (phage) is an intracellular viral-like parasite that infects only one specific bacterial species. Hence, phages can be useful for the identification of bacterial contaminants in food, water, environment, etc. Phages adsorb to specific regions of the

bacterial cell envelope and then the phage genome enters the bacterial cell. The specificity of adsorption of phage to a host is a phenomenon that can be utilized in a phage based biosensor [2]. Phages can attach to proteins, lipopolysaccharides (LPS), pili, and lipoprotein presented on the outer membrane of the bacterial cell. Lytic or virulent phages can multiply in bacteria and kill the cell by lysis at the end of the life cycle, due to the accumulation of a phage lysis protein, and thus, intracellular phages are released into the medium. This specific selectivity of the phage can be used for constructing a sensitive biosensor for bacteria, thereby precluding the need for time consuming conventional microbiological pretreatments. The linkage of phage-specific identification and the release of the inner enzymatic cell markers after the lysis of the cell provide a powerful tool as a highly specific detection method of a given bacterial strain [1].

Phage based biosensors vis-à-vis other detection methods

The lack of specificity is a major drawback in the application of both the AK and ATP assays [45]. Phages were used for the specific lysis of the cells and then subsequent release of ATP while designing an ATP-bioluminescencce assay for *Listeria monocytogenes* which is a food-borne pathogen. Bacteriophage lysis combined with ATP detection enabled the identification of 2.5 X 10^5 cells of *L. monocytogenes* with a signal/background ratio of 10:1 in the presence of an equal number of *L. innocua* cells. The method involved selective pre-enrichment of *Listeria* from food samples before the bacteriophage mediated ATP detection step. The total assay time was 15h with the phage/ATP bioluminescence step taking 80 minutes to perform [36]. A phage-based biosensor was developed by causing the release of intracellular enzymes from bacteria with the help of specific phage to produce a highly specific marker for the bacterial strain *E. coli* K-12, MG1655. The virulent phage l vir serves not only as the specific recognition element for *E. coli* but also as the releasing agent of the enzyme β-d-galactosidase (which is widely used for identifying *E. coli* in water and food samples). The product of its enzymatic activity is measured amperometrically by monitoring its oxidation at the carbon anode. The amperometric detection enables the use of a wide range of bacteria concentrations, reaching as low as 1 cfu / 100 ml within 6–8 hours. The electrochemical method can be applied to any type of bacterium–phage combination by measuring the enzymatic marker released by the lytic cycle of a specific phage [32]. A similar system was also developed for the detection of *Bacillus anthracis* and

Mycobacterium tuberculosis, electrochemically monitoring the catalytic conversion of enzyme substrates released by phage specific cell lysis [46].

Another study conducted to improve the specificity of the AK assay by using phages to lyse target bacteria [3]. The concomitant release of intracellular AK was measured using the bioluminescent method. This approach allowed fewer than 10^3 cfu/ ml *E. coli* to be detected in less than 1 h. However, the lower sensitivity of the phage-mediated bacterial AK assay in comparison with the nonspecific AK assay in this study indicated that release of AK by phage was not optimized and did not reach the maximum level. With this in mind, a study was designed to find out the effect of phage concentration on the activity of AK released from the cells lysed during infection in order to optimize a bioluminescent phage mediated method for bacterial enumeration. It was found that the release of AK was greatest at a multiplicity of infection (moi) of 10-100. It was also concluded that the amount of AK released from *Salmonella enteritidis* and *E. coli* G2-2 cells by specific phages, SJ2 and AT20, respectively, depended on the type of bacteria, the stage of growth, the nature of phage, multiplicity of infection and time [45].

A phage based label-free biosensor was developed for cancer cell monitoring in order to overcome the limitations of the traditional antibody based methods. The antibody based methods are costly and involves difficulty in obtaining and preserving antibodies, on the other hand, phage based methods are robust, highly specific with high yield capacity and low cost. A phage-modified Light Addressable Potentiometric Sensor (phage-LAPS) has been designed which demonstrated that the phage based system is more applicable for detection of cancer cell than cancer biomarkers [22].

A study was carried out to investigate the potential to utilize phage-displayed peptides as reagents in sensor applications. A library of random 12-mers displayed on phage was panned against *Staphylococcal enterotoxin* B (SEB), causative agent of food poisoning. Binding of several of these phages was shown to be inhibited when they were assayed in a competitive enzyme-linked immunosorbent assay (ELISA) format with synthesized peptide corresponding to the peptide-encoding region of one of the clones. Whole phage were labeled with the dye Cy5, and incorporated into fluoro-immunoassays. Labeled phages were able to detect SEB down to a concentration of 1.4 ng/well in a fluorescence-based immunoassay. When incorporated into an automated fluorescence-based sensing assay, Cy5-labeled phage bound to probes coated with

SEB generated a robust signal of about 10,000 pA, vs a signal of 1000 pA using a control fiber coated with streptavidin [16]. Another study used the phage displayed antibodies against the virulence factor actin polymerization protein (ActA) in a Surface Plasmon Resonance (SPR) sensor for biorecognition of whole cells of *Listeria monocytogene*s [31].

Conclusion

Application, effectiveness and drawbacks of various *P. aeruginosa* detection methods have been reviewed critically. All the methods discussed here have their own limitations in one way or the other as no method is perfect in all aspects. Conventional bacterial testing methods besides being labor intensive rely on specific media to enumerate and isolate viable bacterial cells from samples. It often requires a complete series of tests before confirming the identity of a pathogen. It has been reported that bacterial identification using instruments commonly involves: counting the cells by microscope or by flow cytometry; measuring physical parameters by piezo-crystals, impedimetry, redox reactions, optical methods, calorimetry, ultrasound techniques and detecting cellular compounds such as ATP (by bioluminescence), DNA, protein and lipid derivatives (by biochemical methods), radioactive isotopes (by radiometry) [20]. Latest state-of-the-art technologies being developed for rapid detection of micro-organisms include biosensors. Biosensor is an analytical device that couples microorganisms with a transducer to produce a signal proportional to the analyte concentration to enable rapid, accurate and sensitive detection of target analytes in fields as diverse as medicine, forensics, environmental monitoring, defense, food processing, biological warfare and safety. The specificity of adsorption of phage to a host i.e. bacteria is a phenomenon that is being utilized in phage based biosensors [11]. The main advantage of phage based biosensors for bacterial detection is that they provide real-time, on-site detection and analysis in the field and often eliminate the need for sample collection, preparation, and laboratory analysis.

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