Biosensors and Bioelectronics 79 (2016) 213–219

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A luminescent hybridoma-based biosensor for rapid detection of *V. cholerae* upon induction of calcium signaling pathway

Parichehr Zamani [a], Reza H. Sajedi [a,*], Saman Hosseinkhani [a], Mehdi Zeinoddini [b], Bita Bakhshi [c]

[a] Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University,Tehran, Iran
[b] Department of Bioscience and Biotechnology, Mallek Ashtar University of Technology, Tehran, Iran
[c] Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

ARTICLE INFO

Article history:
Received 2 October 2015
Received in revised form
5 December 2015
Accepted 10 December 2015
Available online 11 December 2015

Keywords:
Biosensor
Vibrio cholerae
CANARY technology
Aequorin
Hybridoma cell
Stable cell line

ABSTRACT

In this study, a hybridoma based biosensor was developed for rapid, sensitive and selective detection of *Vibrio cholerae* O1 which converts the antibody–antigen binding to bioluminescence light. After investigation on hybridoma performance, the biosensor was constructed by transfecting specific hybridoma cells with aequorin reporter gene and the bioluminescence activities of stable biosensor were measured. The sensitivity of biosensor was as few as 50 CFU/ml and it showed no responses to other entric bacteria. Moreover, the response time of biosensor was estimated in 7th second which means this method is considerably faster than many available detection assays. In addition, this biosensor was successfully applied to *V. cholerae* detection in environmental samples with no significant loss in sensitivity, demonstrating our proposed biosensor provides a sensitive and reliable method for detection of *V. cholerae* in natural samples. The application of whole hybridoma cell directly as a sensing element in biosensor construction which mentioned for the first time in present study suggests that hybridoma cells could provide a valuable tool for future studies in both basic and diagnostic sciences and could be considered as a fast and specific sensing element for detection of other pathogens in different applications.

1. Introduction

The rapid biological detection using cell based biosensors (CBBs) have already emerged as sensitive and specific tools in biomedical applications as well as in clinical, agricultural and environmental industries (Banerjee and Bhunia, 2009; Banerjee et al., 2013). The conventional recognition methods, routinely used for pathogens, toxins and threat agents like plating methods, immunochemical or molecular based methods often take several hours or days to gain conclusive results (Banerjee and Bhunia, 2009; Stenger et al., 2001). CBBs show the efficient aspects of a host–analyte interaction and render an accurate determination of the risks and analyses different agents employing prokaryotic and eukaryotic cells (Banerjee and Bhunia, 2009; Kintzios, 2005; Pancrazio et al., 1999; Ziegler, 2000). The cellular analysis and notification of antigen risk and yields (CANARY) sensor, expressing specific antibody for particular agents has been considered in developing CBBs (Petrovick et al., 2007; Rider et al., 2003). This diagnostic technology is based on engineered B cells, the fastest pathogen identifiers with intrinsic response in less than 1 s (Petrovick et al., 2007). Expression of aequorin as a calcium sensitive bioluminescent photoprotein and specific antibodies for particular pathogens enable engineered cells to emit light within seconds of exposure to specific analytes (Banerjee and Bhunia, 2009; Stenger et al., 2001). Taking a broader view, binding of antibody to specific antigen initiates a downstream signal transduction cascade that increases cytosolic calcium from both internal and extracellular stores, causing aequorin to emit photons (Curtis et al., 2008; Petrovick et al., 2007; Rider et al., 2003). This biologically inspired technology commercialized for military, clinical and food diagnostic applications, provides rapid identification of pathogens in food, air and medical samples, as well as soluble protein toxins, nucleic acid sequences, viability assessment and antibiotic resistance screening (Banerjee and Bhunia, 2009; Petrovick et al., 2007; Rider et al., 2003). This sensor could detect as few as 50 colony forming unit (CFU) of *Yersinia pestis* in a total assay time of less than 3 min. Moreover, the sensitivity of a B cell line specific for VEE virus is currently 5×10^5 PFU. In addition, 500 CFU/g of *Escherichia coli* strain detected in lettuce in less than 5 min including the initial sample preparation time. Also, CANARY can detect as

* Corresponding author.
E-mail address: sajedi_r@modares.ac.ir (R.H. Sajedi).

http://dx.doi.org/10.1016/j.bios.2015.12.018
0956-5663/

few as 1000 CFU of *Bacillus anthracis* spores extracted from seeded nasal swabs (Petrovick et al., 2007; Rider et al., 2003).

Hybridomas are known as cells derived from B lymphoblasts have been engineered to produce a desired antibody in large amounts (Pandey, 2010). The synthesis of monoclonal antibodies from hybridoma cells established in 1975 by Kohler and Milstein has led to a revolution in the biological sciences (Milstein, 1999). The applications of these antibodies isolated from various hybridoma cells are numerous, involving diagnostic, therapeutic and analytical purposes (Kramer and Hock, 2003; Pandey, 2010). Previously, isolation of monoclonal antibodies against *Vibrio cholerae* from a collection of hybridoma cell lines was reported by Robb et al. (1982), but our study presents the first investigation of sensing system using whole hybridoma cell directly for detection of *V. cholerae* which is a natural inhabitant of the aquatic environments and establishes the cause of life-threatening epidemics in developing countries including Iran (Bakhshi and Pourshafie, 2009; Colwell et al., 1977; Islam et al., 1994). The diarrheal disease cholera in the epidemic form is caused by *V. cholerae*, belonging to the O1 or O139 serogroup (Kaper et al., 1995). The non-O1, non-O139 serogroups being isolated in abundance from aquatic or estuarine sources, has been associated with limited gastroenteritis in humans (Morris, 1989). Early diagnosis and confirmation of *V. cholerae* is crucial for rapid implementation of control measures. Different conventional cultural, molecular, biochemical and immunological assays are used for *V. cholerae* detection with sensitivity of about 10–10^6 CFU, however, many of these procedures currently used for *V. cholerae* detection are time consuming and most of them are not adequate to identify low numbers of this bacterium (Barzamini et al., 2014; O'Hara et al., 2003; Yadava et al., 2014). This report describes the development of a simple, sensitive and specific method for detection of *V. cholerae* using hybridoma cells containing antibody against *cholerae* O1 antigen instead of engineered B cells used in CANARY technology. Although there are many published methods, such as those mentioned above, the trend has become towards the development of faster detection assay distinguishing a few CFU of this bacteria.

2. Materials and methods

2.1. Cell culture

Hybridoma cells producing IgM antibody against LPS of *V. cholerae* O1 were provided using fusion of immunized spleen cell with mouse myeloma SP2/0 (produced in Avicenna Research Institute, MARC, Tehran, Iran). In addition, mouse myeloma SP2/0 and MC2B8 hybridoma (cell line producing monoclonal antibody against plasminogen) used as controls for following assays. The cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 units/mL of penicillin and 100 μg/mL of streptomycin at 37 °C in 5% CO_2. They were then transferred to medium containing 10% DMSO, frozen in liquid nitrogen for long-term storage. Cells were grown to approximately 80% confluence in prepared medium for following assays.

2.2. Immunocytochemistry

Flat-bottomed 96 well cell culture plates were used. Each well received 150 μl of the appropriate bacterial suspension in carbonate buffer (equivalent to 10^3 bacteria per well). This concentration was selected after microscopic examination of wells coated with different concentrations of bacteria per well (10–10^4 CFU) using Olympus-IX81 (Olympus Optical, Tokyo, Japan) inverted microscope. The wells left overnight for adsorption process at 4 °C, the plates were dried and washed with phosphate-buffered saline (PBS; pH 7.4) containing 0.1% (v/v) Tween 20. After 2 times washing, the remaining binding sites on the plates were blocked with PBS containing 10% (w/v) BSA (1 h, 37 °C) (Elder et al., 1982). The selected cells were stained using acridine orange metachromatic stain with some modifications according to Belyaeva et al. (2009) method. Hybridoma cells were centrifuged at 326*g* for 10 min, and washed twice with 1 ml of with PBS (pH 7.4). Then, cells were incubated in 0.1 mg/ml acridine orange for 10 min. After two more washes, cells were added to bacteria-coated wells as follows. The assay was based on that of Voller et al. (1976). 200 μl of cellular suspensions (3×10^5 cells) in PBS containing 0.1% (v/v) Tween 20 was added to the bacteria-coated wells and incubated for 1 h at room temperature. After three washes steps, cell bound to bacteria were analyzed with two separate blue and green filters using an inverted microscope, Olympus IX81 equipped with a digital camera (Olympus, 72 DPI). In addition, acridine orange fluorescence emission on 525 nm was recorded using Synergy Multi-mode Reader (BioTek, USA). The specificity of the assay was evaluated by using SP2/0 and MC2B8 hybridoma cell lines as negative controls.

2.3. Intracellular calcium measurement

Cells were washed with PBS and dye loaded using 2 μM of fura 2-AM for 30 min at 37 °C in the dark. Following extensive washing, the cells were incubated for a further 30 min to allow complete ester hydrolysis at 37 °C. After washing, cells were exposed to 500 CFU of *V. cholerae*, then read using Synergy Multi-mode Reader (BioTek, USA).

2.4. Construction of hybridoma cells stably expressing aequorin

Hybridoma cells, grown to 70% confluence, were transfected with pBudCE 4.1 plasmid encoding aequorin gene by lipofectamine (Life technologies, USA) according to the recommended procedures by the manufactures with the final concentration of 2 μg DNA per well. Before transfection, untransfected cells were treated by different concentration of zeocin (25–1000 μg/ml) to estimate the minimum concentration that kills these cells in 2 weeks. After 3 days of transfection, cells were treated by 100 and 200 μg/ml zeocin for 1 and 2 weeks respectively. Then, zeocin concentration increased to 300 μg/ml for over 4 months.

2.5. Evaluation of aequorin in transfected hybridoma cells

2.5.1. SDS–PAGE and western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) carried out using a 12% (w/v) polyacrylamide gel by the method of Laemmli (1970). For blotting, the gel was transferred onto the nitrocellulose membrane. Then, aequorin identified by reaction with polyclonal primary (Zeinoddini et al., 2013) and secondary antibodies linked to horseradish peroxidase. Reactive bands were detected immunochemically according to the enhanced chemiluminescence (ECL) western blotting protocol (Amersham) and chemiluminescence was visualized with charged-coupled devices (CCD camera).

2.5.2. Aequorin assay in cell lysate

Transfected cells were homogenized in ice-cold lysis buffer as mentioned in supplementary data. For determining the luminescence activity of aequorin, cells were incubated for 2 h with 10 μM coelenterazine and the regenerated aequorin was injected with 50 mM $CaCl_2$ in 50 mM Tris–HCl (pH 7.6) (Inouye and Sahara, 2007). The luminescence activity was measured using Synergy Multi-mode Reader (BioTek, USA).

Details on cell lysate preparation, PCR analysis and more information on western blotting are provided in supplementary data.

2.6. Determination of biosensor activity

Biosensor activity was determined by measuring the aequorin light release evoked by treatment of stably transfected cells with *V. cholerae* according to Bovolenta et al. in 96-well plates with slight modifications (Bovolenta et al., 2007). Cells were incubated with 10 μM coelenterazine for 2 h at 37 °C, and, after adding different CFU of *V. cholerae*, the produced light with Synergy Multi-mode Reader (BioTek, USA) was measured.

2.7. Investigation on biosensor specificity

In order to evaluate biosensor specificity, the biosensor was prepared as mentioned above and exposed to 1000 CFU of other enteric bacteria including *Salmonella typhi*, *Shigella sonnei*, *E. coli*, *Entrobacter cloacae*, *Citrobacter freundii* and *Pseudomonas aeruginosa* and the luminescence was recorded. More details on bacterial strains and growth conditions are described in supplementary data.

2.8. Environmental and clinical sample analysis

Environmental sample analysis carried out with water samples from fresh water, as well as wastewater and sea water spiked with serial dilutions of *V. cholerae*. The CFU of bacteria added to spike the water was determined by plating serial dilutions of the initial inoculum. In addition, for providing spiking stool, a faecal specimen from a healthy individual was diluted 5-fold with physiologic sodium chloride solution followed by addition of different CFU values of bacteria and determination of biosensor activity as mentioned above.

3. Results

3.1. Whole-bacterial cell-linked immunosorbent assay

The ability of hybridoma cell line in binding to *V. cholerae* O1 lipopolysaccharide was investigated by the immunocytochemistry method using acridine orange. For this approach, 0.1 mg/ml dye was added to the culture medium and fluorescence imaging was captured using Olympus-IX81 microscope. Fig. 1A shows images captured with the blue and green filters separately after exposure of stained cells to bounded *V. cholerae* in 96 well plate. The data showed that specific hybridoma remained as attached to *V. cholerae* after 3 times washing which demonstrate the existence of

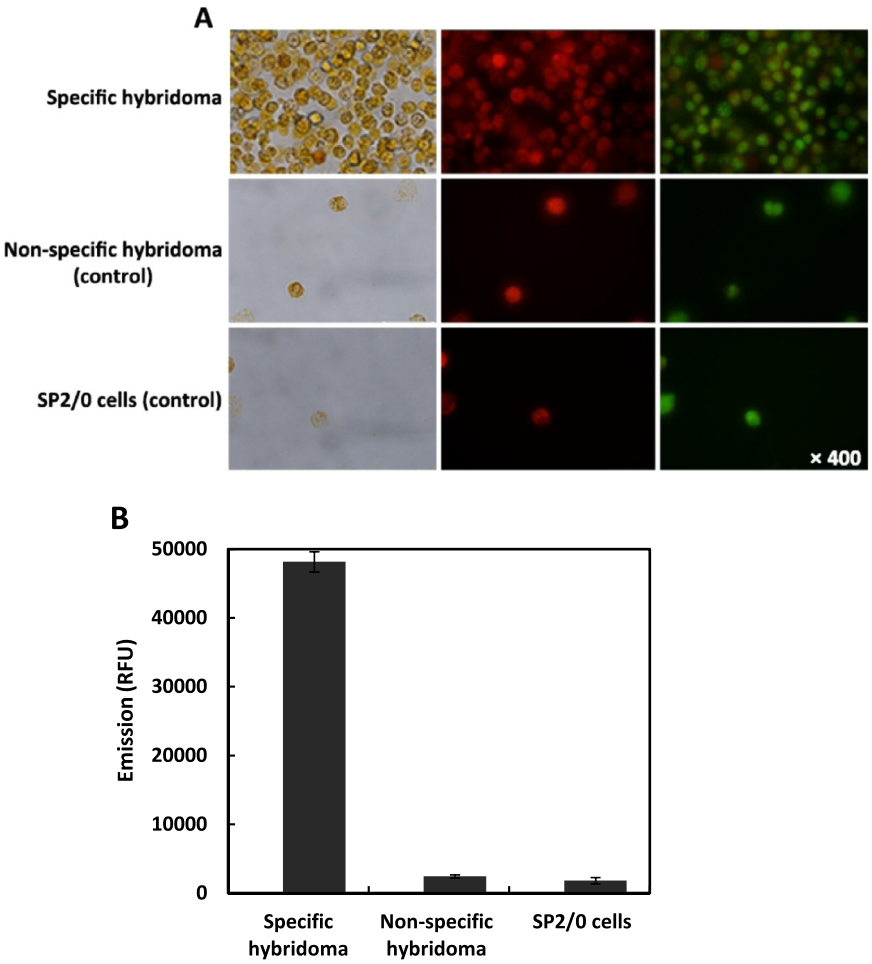

Fig. 1. Whole bacterial cell-linked immunocytochemistry. Cell bound to *V. cholerae* was analyzed after staining cells with acridine orange followed by addition of stained cells to bacteria-coated wells; SP2/0 and MC2B8 hybridoma cell lines used as controls. (A) After 3 times washes, just specific hybridoma remains attached to *V. cholerae* observed by optical and fluorescence microscopy. Also, the cells were seen green and red using blue and green filters respectively. (B) Acridine orange fluorescence intensity was determined by recording emission on 525 nm in exciting wavelength of 502 nm. An obvious rises in emission intensity which just occurred in specific hybridoma confirmed the microscopic results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

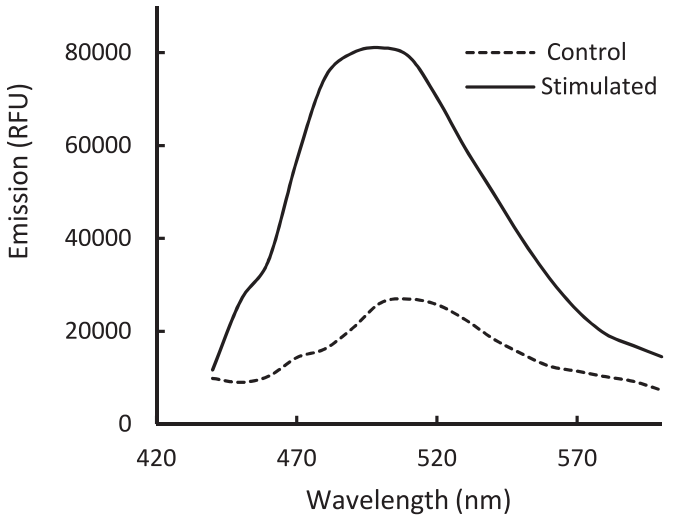

Fig. 2. Measurement of intracellular calcium released in hybridma cells. Fluorescence spectrum was plotted by loading cells with 2 μM of fura 2-AM for 30 min in dark measured by excitation at 340 nm. Binding of antibody to specific antigen initiates a signal transduction cascade that increases cytosolic calcium. The emission spectrum of fura 2-AM as a sensitive calcium indicator was significantly increased at 505 nm.

specific O1 antibody on the cell surface which efficiently binds to its antigen. No other cell lines including SP2/0 and MC2B8 stayed attached to *V. cholerae* after washing steps which shows high-specificity of this hybridoma cells for *V. cholerae*. In addition, acridine orange fluorescence was investigated by recording emission on 525 nm upon excitation at 502 nm. The fluorescence emission of acridine orange has been used to follow binding of antigen to its specific antibody. As shown in Fig. 1B, high-emission of specific hybridoma attached to *V. cholerae* confirmed the microscopic results.

3.2. Measurement of intracellular calcium released in specific hybridoma cells

The elevation pattern of intracellular calcium was measured by a calcium fluorescence indicator; fura 2-AM after *V. cholerae* addition. As depicted in Fig. 2, after binding of specific hybridoma cells to *V. cholerae,* the emission spectra was measured by fluorescence excitation at 340 nm. The emission of fura 2-AM was significantly increased at 505 nm due to binding of released calcium to fura 2-AM at the presence of *V. cholerae*.

3.3. Preparation of stable biosensor

The results of cloning and sequencing revealed 630 bp aequorin gene was successfully ligated into the *Kpn*I and *Xho*I sites of plasmid pBudCE4.1 (Supplementary data). Hybridoma cells containing membrane-bound antibody against *vibrio* O1 antigen were transfected with pBudCE4.1 expression plasmid encoding aequorin and they were maintained at high-concentration of zeocin. However, untransfected cells were completely killed in media containing 200 μg/ml zeocin, transfected cells were cultured in the presence of 300 μg/ml zeocin for several months to produce stable cell line. The evaluation of aequorin in transfected hybridoma cells is described in supplementary information.

3.4. Biosensor specification

Hybridoma cells stably expressing functional aequorin were selected for determination of the optimal response to *V. cholerae* (Fig. 3). The bioluminescence spectrum, shown in Fig. 4A, confirm

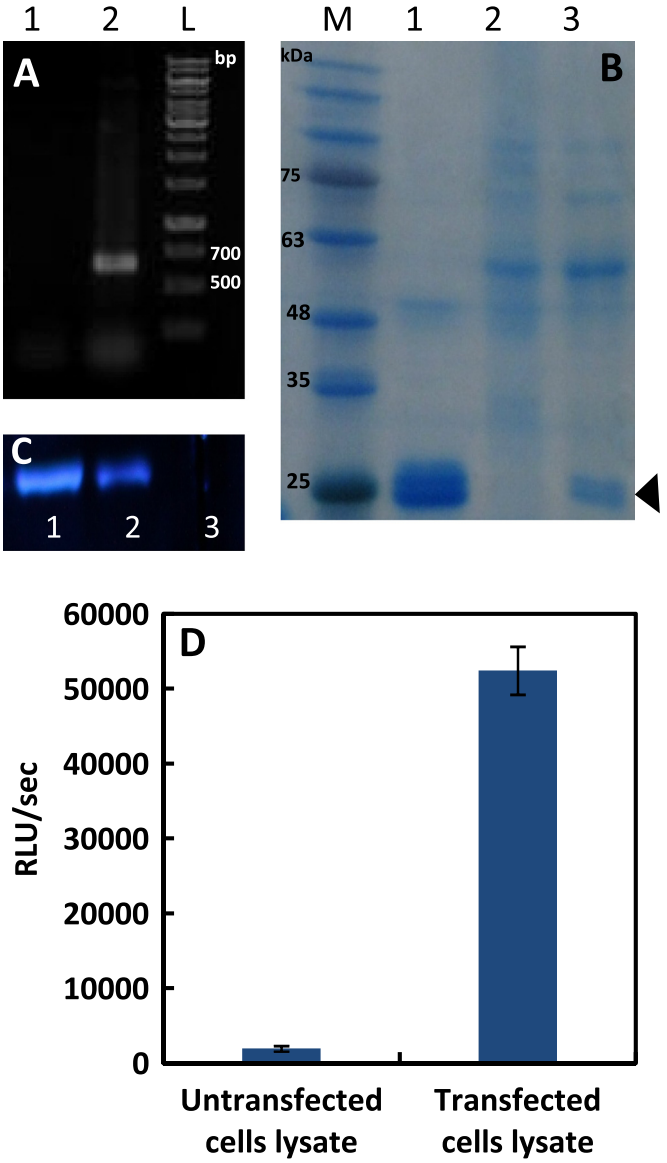

Fig. 3. Expression of aequorin in transfected cells. Specific hybridoma cells were transfected with recombinant vector (pBudCE4.1 plasmid encoding aequorin); untransfected cells were taken as control. (A) PCR data showed a 630 bp aequorin coding gene in transfected cells, no band were observed in PCR of untransfected cell, lane 1, control; lane 2, transfected cells; lane L, DNA ladder. (B) SDS–PAGE of transfected cell lysate showed an about 22 kDa band related to aequorin compared to untransfected cells, lane M, molecular size markers; lane 1, aequorin; Lane 2, control; lanes 3, transfected cells. (C) Immunoblotting of transfected and untransfected cells were performed for investigation of aequorin expression in transfected cell. An obvious chemiluminescence band appeared in transfected cell. Lanes 1, purified aequorin as control; lane 2, transfected cells; lane 3, untransfected cells. (D) *in vitro* reconstitution of aequorin was determined by exposing cell lysates of transfected cells to solutions with known calcium concentrations. Luminescence activity in transfected cell lysates confirmed expression and regeneration of aequorin in transfected cells. The data are means ± SD of triplicate experiments.

emission of blue light (λ_{max}=465 nm). Moreover, different parameters were analyzed in a wide range of conditions provided in supplementary data to improve the luminescence signals (Figs. S2 and S3). The detection time of biosensor in the presence of 200 CFU of *V. cholerae* have been shown in Fig. 4. Significant increase in light emission started about 7 s after adding *V. cholerae* to specific biosensor (Fig. 4B), and maximum emission was observed after 12 sec, which means that, this method can detect *V. cholerae* in less than 20 s. This demonstrates the sensor's potential to rapid screening of *V. cholerae*. In addition, no significant differences were

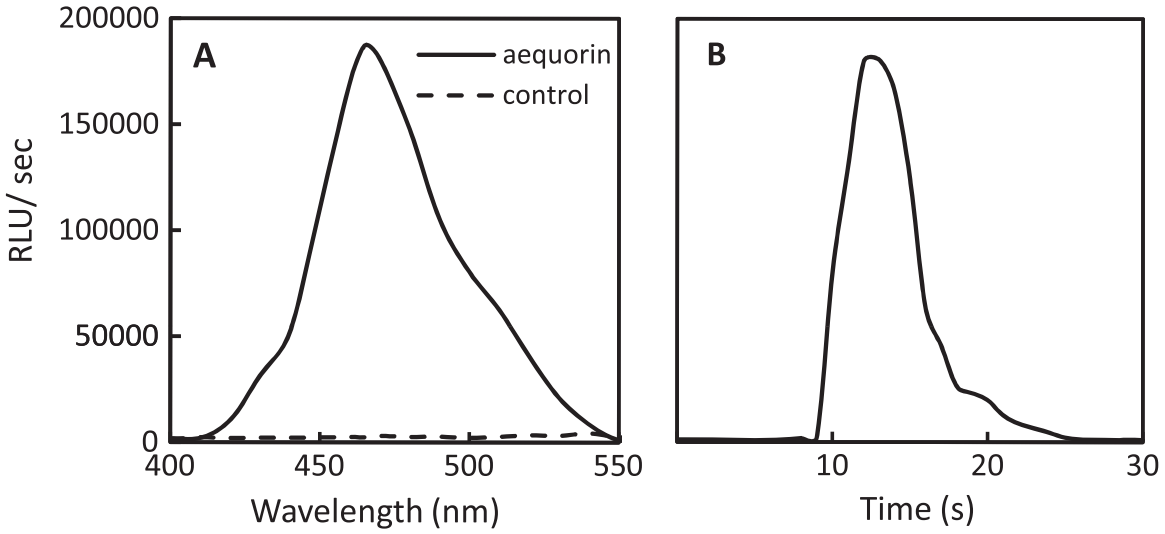

Fig. 4. Determination of biosensor activity in response to *V. cholerae*. (A) The bioluminescence emission spectrum of aequorin in transfected cell showed a maximum intensity at 465 nm after adding the bacteria. (B) Detection time of biosensor revealed that significant increase in light emission started in 7th second after *V. cholerae* addition to the transfected cells and the maximum bioluminescence emission was obtained after 12 s.

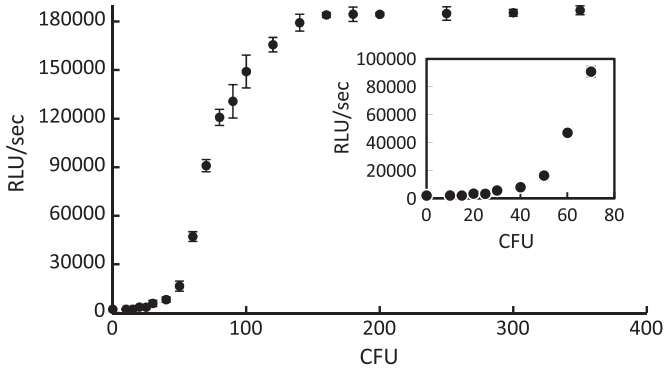

Fig. 5. Sensitivity of biosensor in response to *V. cholerae*. Up to 10^3 CFU/ml of *V. cholerae* were added to 3×10^5 specific hybridoma cells in the assay plate and the luminescence activity was measured. Dose dependent curve showed that constructed biosensor detected as few as 50 CFU/ml (12 CFU per assay) of *V. cholerae*. Also, EC_{50} was calculated about 74 CFU/ml.

found in detection time of biosensor exposed to different CFU of *V. cholerae* (not shown data). For determining biosensor sensitivity, 3×10^5 specific hybridoma cells was added to the assay plate followed by injection of *V. cholerae* in the range 0–10^3 CFU/ml. As shown in Fig. 5, a significant level of photon emission was monitored as the result of the *V. cholerae* interaction with constructed biosensor and specific cells detected as few as 50 CFU/ml of *V. cholerae* (12 CFU per assay). Moreover, the EC_{50} was measured about 74 CFU/ml (Fig. 5).

3.5. Biosensor specificity and its activity in environmental samples

The specificity of biosensor for *V. cholerae* was determined by exposure of a collection of enteric bacteria to designed biosensor. No significant responses were observed at the presence of other bacteria, but light emission in the presence of *V. cholerae* was increased significantly (Fig. 6A). The biosensor activity in different environmental and clinical samples was investigated. The detection limit of the assay was determined to be 70 CFU/ml in stool samples. Also, this assay was successfully performed for detection of *V. cholerae* from environmental water samples in the order of 50 CFU/ml and there was no significant difference in biosensor activity for fresh water, wastewater and sea water. The maximum luminescence emission was obtained when samples spiked with 150, 160 and 200 CFU/ml of *V. cholerae* in fresh water, sea water and stool sample respectively (Fig. 6B). The validity of the designed biosensor was tested by culture method and similar results were obtained from both tests.

4. Discussion

In this study, a sensitive, specific and rapid method was proposed for *V. cholerae* detection based on CANARY technology. The uniqueness of this constructed biosensor is its sensing element, which is specific hybridoma cell for *V. cholerae* O1 antigen and it is able to show analyte interaction with no additional processing including isolation of antibodies or transfecting antibody genes to B lymphocytes which applied in CANARY biosensor. For this approach, whole bacterial cell-linked immunocytochemistry were examined using acridine orange to evaluate hybridoma efficiency. Both microscopic imaging and acridine orange fluorescence analysis in O1 specific hybridoma displayed antibodies on its surface as pathogen receptors (Fig. 1). For calcium detection after antigen binding, hybridoma cell was exposed to fura 2-AM which showed an increase in emission intensity at 505 nm in the presense of *V. cholerae*. From these results it can be concluded that intracellular calcium concentration was significantly increased in hybridoma cells (Fig. 2). The obtained data revealed that hybridoma cells could be successfully applied instead of engineered B cells in CANARY technology. The specific hybridoma cells were transfected with recombinant plasmid encoding aequorin reporter gene and maintained in high-concentration of antibiotic to achive a stable biosensor. After optimizing of biosensor conditions, the sensitivity of the biosensor was evaluated using 3×10^5 cells/well in a 96-well plate (Fig. S2). The maximum bioluminescence response was obtained after 12 s of stimulating with *V. cholerae* (Fig. 4B). Also, the detection limit of the assay was determined to be as few as 50 CFU/ml (12 CFU per assay) (Fig. 5) which shows the potential of this biosensor for rapid and sensitive screen of this bacteria.

To date, detection of *V. cholerae* is typically carried out in different environments with various methods, including an immunofluorescent-aggregation (IFAG) assay (Wang et al., 2010), biochemical tests (Bakhshi, et al., 2008; Baumann, 1984; Choopun et al., 2002; Iii et al., 1985; Kay et al., 1994; West, 1984), molecular methods including PCR, real time PCR, reverse transcription PCR, conventional and multiplex PCR, DNA–DNA hybridization (Alam

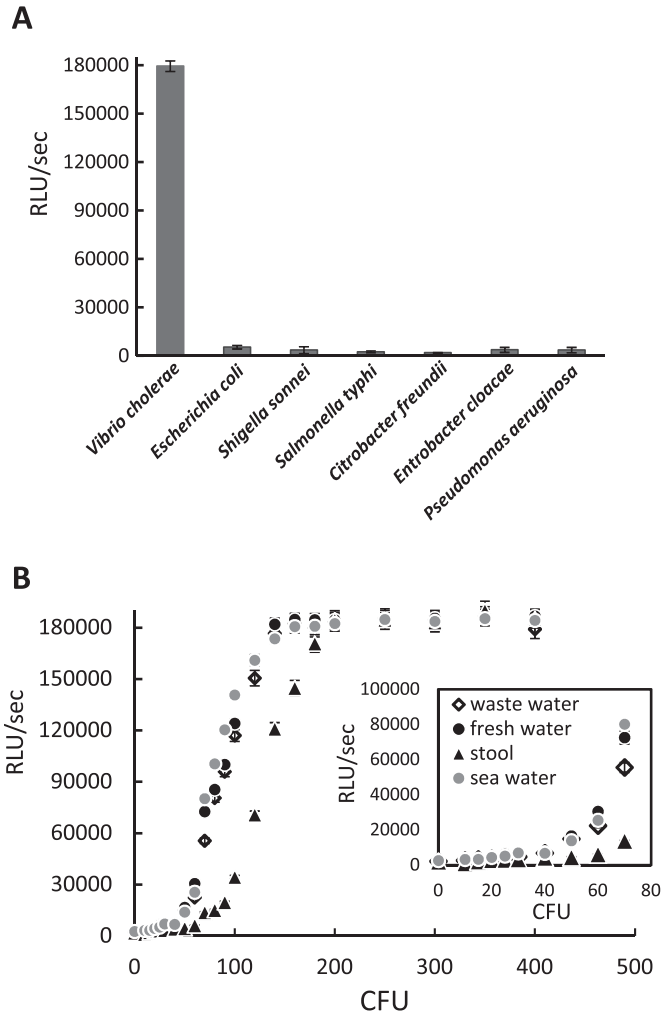

Fig. 6. Biosensor selectivity and its sensitivity in different environmental samples. (A) The selectivity of biosensor to *V. cholerae* was determined by exposure of various bacteria (1000 CFU/ml) to constructed biosensor. No significant bioluminescence emission recorded in the presence of other bacteria compared to *V. cholerae*, indicating the high-selectivity of biosensor to this bacterium. Values are mean ± SD of three separate experiments. (B) Environmental sample analysis carried out with water samples (fresh water, wastewater and sea water) and stool sample spiked with different serial dilutions of *V. cholerae* (including 0–1000 CFU/ml). The limit of bacterial detection for spiked environmental water and stool samples were as few as 50 and 70 CFU/ml, respectively.

et al., 2010; Bej et al., 1996; Chun et al., 1999; Imani et al., 2013; Indrawattana et al., 2014; Khemthongcharoen et al., 2015; Lipp et al., 2003; Nandi et al., 2000; Zeinoddini et al., 2014), a loop-mediated isothermal amplification (LAMP) (Yamazaki et al., 2008), immunoassay using specific monoclonal antibodies (Adams et al., 1988; Hasan et al., 1994), dipstick test (Chakraborty et al., 2013; George et al., 2014; Nato et al., 2003), intrinsic catalytic activity of a magnetic polymeric nanoparticle (Thiramanas et al., 2013) and application of biocompatible capped iron oxide nanoparticle (Sharma et al., 2015). Among these, reverse transcription and real time PCR are considered as the most accurate methods; however, in order to obtain a final diagnosis, a considerable amount of time is needed. The sensitivity of PCR assay in various samples including cultured colonies, environmental water and stool samples was usually estimated about 10^2–10^6 CFU/ml in most studies (Barzamini et al., 2014; Gubala, 2006; Nhung et al., 2007; O'Hara et al., 2003; Panicker et al., 2004; Senachai et al., 2013; Yadava et al., 2014; Zeinoddini et al., 2014). Moreover, the limit of detection decreased to 1 CFU/10 g of spiked cockle tissue and 1 CFU/g in oyster samples after 5–6 h of enrichment prior to PCR (Senachai et al., 2013). The minimum detection limit of the real time PCR for *V. cholerae* detection in environmental water sample was 1.4 CFU/ml reported by Bielawska-Drozd et al. (2012). The results of electrochemical study of biocompatible capped iron oxide nanoparticles reported by Sharma et al. (2015) exhibits a detection limit of 0.32 ng/ml (Sharma et al., 2015). Yamazaki et al. (2008) reported that 7.8×10^2 CFU per g (1.4 CFU per reaction) required in the process of 35 and 70 min using a colony on thiosulfate citrate bile salts sucrose (TCBS) agar and spiked human feces respectively in LAMP assay (Yamazaki et al., 2008). The limit of detection in immunofluorescent aggregation (IFAG) assay was determined to be 10^3 CFU/ml (Wang et al., 2010). In Dipstick Tests, a minimum of 10^6–10^7 CFU of *V. cholerae* O1/ml is required to give an unequivocal positive reaction in stool samples (George et al., 2014; Mukherjee et al., 2010; Nato et al., 2003). A study reported by Chakraborty et al. (2013) detected samples spiked with < 10 CFU after 24 h and ~200 CFU after 6 h of incubation at 37 °C in environmental water. In addition, detection of *V. cholerae* in all spiked specimens was negative at 2 h and 4 h of incubation using this method (Chakraborty et al., 2013). To study the biosensor specificity, different bacteria were exposed to *V. cholerae* biosensor. As shown in Fig. 6A, no bioluminescence emission was detected for other enteric bacteria which indicates the high-specificity of biosensor for *V. cholerae*. A vast array of gene targets such as *hlyA*, *zot*, *ompU* and *tox* have been used in PCR detection assays for *V. cholerae* (Barzamini et al. 2014; Rivera et al., 2001; Zeinoddini et al., 2014). This variation in target genes was due to lack of reliable results for accurate detection of *V. cholerae* in application of similar gene sequences (Boyd et al., 2000; Di Pinto et al., 2005). In addition, a selective medium, such as thiosulfate citrate bile salts sucrose (TCBS) agar which is considered as other conventional method has the same limitations for environmental samples because many other bacteria present in natural sources grow with an appearance similar to *V. cholerae* colonies (Choopun et al., 2002). Also, recent studies of the crystal VC dipstick tests resulted in low specificities (George et al., 2014) such as 50.8% of false positive dipstick tests in study of Ley et al. (2012). In the present study, the base of the assay is specificity of antibody to its antigen which lead to the high-specificity of of the proposed method in *V. cholerae* detection, which can be tailored to a desired application. Finally, the developed biosensor was successfully applied for *V. cholerae* detection in environmental spiked water and stool samples with no significant decrease in sensitivity. The detection limit of assay in spiked environmental water and stool samples were as few as 50 and 70 CFU/ml, respectively (Fig. 6B). According to the reported results of other literatures, efforts for detecting *V. cholerae* directly in environmental samples have previously been limited by laboratory testing particularly PCR-based experiments (Chakraborty et al., 2013; Gubala, 2006). Sea water samples causing inhibitory effect on PCR reagents, probably due to the high salt concentration as well as other organic substances in environmental water samples (Gubala, 2006). To remove inhibitors, some other studies developed the time consuming process involving DNA purification prior to PCR analysis (Gubala, 2006; Lipp et al., 2003; Rivera et al., 2001).

5. Conclusions

In summary, hybridoma cell selected in this study potentially enables detection of *V. cholerae* O1 serotype. The results revealed that the hybridoma cell is a suitable candidate for specific sensing element in biosensor construction and it can be utilized in other diagnostic tools. Also, the speed, sensitivity and specificity of constructed biosensor are valuable attributes for *V. cholerae* detection and it can be considered as a fast and reliable diagnostic

tool for other pathogens, toxins and environmental compounds in different applications.

Acknowledgments

The authors express their gratitude to the research council of Tarbiat Modares University (with the Grant Number of 6236) and Ministry of Sciences, Researches and Technology for financial support during the course of this project. We also thank Dr. Manouchehr Mirshahi for providing the control cell lines used in this investigation.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.12.018.

References

Adams, L.B., Henk, M.C., Siebeling, R.J., 1988. J. Clin. Microbiol. 26 (9), 1801–1809.
Alam, M., Hasan, N.A., Sultana, M., Nair, G.B., Sadique, A., Faruque, A.S.G., Endtz, H.P., Sack, R.B., Huq, A., Colwell, R.R., 2010. J. Clin. Microbiol. 48 (11), 3918–3922.
Bakhshi, B., Pourshafie, M.R., Navabakbar, F., Tavakoli, A., Shahcheraghi, F., Salehi, M., Faradjzadegan, Z., Zahraei, M., 2008. Iran. Biomed. J. 12 (3), 159–165.
Bakhshi, B., Pourshafie, M.R., 2009. Scand. J. Infect. Dis. 41 (4), 256–262.
Banerjee, P., Bhunia, A.K., 2009. Trends Biotechnol. 27 (3), 179–188.
Banerjee, P., Kintzios, S., Prabhakarpandian, B., 2013. Toxins 5 (12), 2366–2383.
Barzamini, B., Moghbeli, M., Soleimani, N.A., 2014. Int. J. Enteric Pathog. 2 (4), e20997.
Baumann, O., 1984. Family II. Vibrionaceae. In: Krieg, N.R., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 1:516-550.
Bej, A.K., Ng, W.-Y., Morgan, S., Jones, D.D., Mahbubani, M.H., 1996. Mol. Biotechnol. 5 (1), 1–10.
Belyaeva T.N., et al., Cell Tissue Biol. 3(2), 2009, 173–180.
Bielawska-Drozd, A., Mirski, T., Bartoszcze, M., Cieslik, P., Roszkowiak, A., Michalski, A., 2012. Pol. J. Environ. Stud. 21, 2.
Bovolenta, S., Foti, M., Lohmer, S., Corazza, S., 2007. J. Biomol. Screen. 12 (5), 694–704.
Boyd, E.F., Moyer, K.E., Shi, L., Waldor, M.K., 2000. Infect. Immun. 68 (3), 1507–1513.
Chakraborty, S., Alam, M., Scobie, H.M., Sack, D.A., 2013. Front. Microbiol. 4.
Choopun, N., Louis, Vr., Huq, A., Colwell, R.R., 2002. Appl. Environ. Microbiol. 68 (2), 995–998.
Chun, J., Huq, A., Colwell, R.R., 1999. Appl. Environ. Microbiol. 65 (5), 2202–2208.
Colwell, R.R., Kaper, J., Joseph, S.W., 1977. Science 198 (4315), 394–396.
Curtis, T., Naal, R.M.Z.G., Batt, C., Tabb, J., Holowka, D., 2008. Biosens. Bioelectron. 23 (7), 1024–1031.
Di Pinto, A., Ciccarese, G., Tantillo, G., Catalano, D., Forte, V.T., 2005. J. Food Prot. 68 (1), 150–153.
Elder, B.L., Boraker, D.K., Fives-Taylor, P.M., 1982. J. Clin. Microbiol. 16 (1), 141–144.
Iii, J.J. Farmer, Hickman-Brenner, F.W., Kelly, M.T., 1985. Vibrio. Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, DC, pp. 282–301.
George, C.M., Rashid, M., Sack, D.A., Sack, R. Bradley, Rahman, K.M. Saif, Azman, A.S., Monira, S., Bhuyian, S.I., Rahman, K.M. Zillur, Mahmud, M. Toslim, 2014. Trop. Med. Int. Health 19 (3), 301–307.
Gubala, A.J., 2006. J. Microbiol. Methods 65 (2), 278–293.
Hasan, J.A.K., Bernstein, D., Huq, A., Loomis, L., Tamplin, M.L., Colwell, R.R., 1994. FEMS Microbiol. Lett. 120 (1–2), 143–148.
Imani, F.A.A., Iman, I.D., Hosseini, D.R., Karami, A., Marashi, S.M., 2013. Asian Pac. J. Trop. Med. 6 (2), 115–118.
Indrawattana, N., Promptmas, C., Wat-Aksorn, K., Soontornchai, S., 2014. Anal. Methods 6 (19), 7634–7639.
Inouye, S., Sahara, Y., 2007. Protein Expr. Purif. 53 (2), 384–389.
Islam, M.S., Drasar, B.S., Sack, R.B., 1994. J. Diarrhoeal Dis. Res. 12 (2), 87–96.
Kaper, J.B., Morris Jr., J.G., Levine, M.M., 1995. Clin. Microbiol. Rev. 8, 48–86.
Kay, B.A., Bopp, C.A., Wells, J.G., 1994. Isolation and identification of Vibrio cholerae O1 from fecal specimens. In: Vibrio Cholerae and Cholera: Molecular to Global Perspectives. ASM Press, Washington, DC. pp. 3–25.
Khemthongcharoen, N., Wonglumsom, W., Suppat, A., Jaruwongrungsee, K., Tuantranont, A., Promptmas, C., 2015. Biosens. Bioelectron. 63, 347–353.
Kintzios, S.E., 2005. Front. Drug. Des. Discov. 2, 225–239.
Kramer, K., Hock, B., 2003. Anal. Bioanal. Chem. 377 (3), 417–426.
Laemmli U.K., 1970 Nature 227(5259), 680-685.
Ley B, et al., 2012 PloS one 7(5), e36930.
Lipp, E.K., Rivera, I.N.G., Gil, A.I., Espeland, E.M., Choopun, N., Louis, Vr.R., Russek-Cohen, E., Huq, A., Colwell, R.R., 2003. Appl. Environ. Microbiol. 69 (6), 3676–3680.
Milstein, Cs, 1999. Bioessays 21 (11), 966–973.
Morris Jr, J.G., 1989. Epidemiol. Rev. 12, 179–191.
Mukherjee, P., Ghosh, S., Ramamurthy, T., Bhattacharya, M.K., Nandy, R.K., Takeda, Y., Nair, G.B., Mukhopadhyay, A.K., 2010. Jpn. J. Infect. Dis. 63 (4), 234–238.
Nandi, B., Nandy, R.K., Mukhopadhyay, S., Nair, G.B., Shimada, T., Ghose, A.C., 2000. J. Clin. Microbiol. 38 (11), 4145–4151.
Nato, F., Boutonnier, A., Rajerison, M., Grosjean, P., Dartevelle, S., Guenole, A., Bhuiyan, N.A., Sack, D.A., Nair, G.B., Fournier, J.M., 2003. Clin. Diagn. Lab. Immunol. 10 (3), 476–478.
Nhung, P.H., Ohkusu, K., Miyasaka, J., Sun, X.S., Ezaki, T., 2007. Diagn. Microbiol. Infect. Dis. 59 (3), 271–275.
O'Hara, C.M., Sowers, E.G., Bopp, C.A., Duda, S.B., Strockbine, N.A., 2003. J. Clin. Microbiol. 41 (12), 5654–5659.
Pancrazio, J.J., Whelan, J.P., Borkholder, D.A., Ma, W., Stenger, D.A., 1999. Ann. Biomed. Eng. 27 (6), 697–711.
Pandey, S., 2010. Hybridoma 1 (2), 017.
Panicker, G., Myers, M.L., Bej, A.K., 2004. Appl. Environ. Microbiol. 70 (1), 498–507.
Petrovick, M.S., Harper, J.D., Nargi, F.E., Schwoebel, E.D., Hennessy, M.C., Rider, T.H., Hollis, M.A., 2007. Linc. Lab. J. 17 (1).
Rider, T.H., Petrovick, M.S., Nargi, F.E., Harper, J.D., Schwoebel, E.D., Mathews, R.H., Blanchard, D.J., Bortolin, L.T., Young, A.M., Chen, J., 2003. Science 301 (5630), 213–215.
Rivera, I.N.G., Chun, J., Huq, A., Sack, R.B., Colwell, R.R., 2001. Appl. Environ. Microbiol. 67 (6), 2421–2429.
Robb, M., Nichols, J.C., Whoriskey, S.K., Murphy, J., 1982. Infect. Immun. 38, 267–272.
Senachai, P., Chomvarin, C., Namwat, W., Wongboot, W., Wongwajana, S., Tangkanakul, W., 2013. Southeast Asian J. Trop. Med. Public Health 44, 249–258.
Sharma, A., Baral, D., Rawat, K., Solanki, P.R., Bohidar, H.B., 2015. Nanotechnology 26 (17), 175302.
Stenger, D.A., Gross, G.W., Keefer, E.W., Shaffer, K.M., Andreadis, J.D., Ma, W., Pancrazio, J.J., 2001. Trends Biotechnol. 19 (8), 304–309.
Thiramanas, R., Jangpatarapongsa, K., Tangboriboonrat, P., Polpanich, D., 2013. Anal. Chem. 85 (12), 5996–6002.
Voller, A., Bidwell, D., Bartlett, A., 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections. In: Rose, N., Feldman, H. (Eds.), Manual of clinical immunology. American Society for Microbiology, Washington, DC, (506 pp).
Wang, D., Xu, X., Deng, X., Chen, C., Li, B., Tan, H., Wang, H., Tang, S., Qiu, H., Chen, J., 2010. Appl. Environ. Microbiol. 76 (16), 5520–5525.
West, P.A., Colwell, R.R., 1984. Identification and classification of Vibrionaceae-an overview. In: Colwell, R.R. (Ed.), Vibrios in the Environment, John Wiley, New York, pp. 285-363.
Yadava, J.P., Jain, M., Goel, A.K., 2014. Water SA 39 (5), 611–614.
Yamazaki, W., Seto, K., Taguchi, M., Ishibashi, M., Inoue, K., 2008. BMC Microbiol. 8 (1), 94.
Zeinoddini, M., Khajeh, K., Hosseinkhani, S., Saeedinia, A.R., Robatjazi, S.-M., 2013. Appl. Biochem. Biotechnol. 170 (2), 273–280.
Zeinoddini, M., Saeedinia, A.R., Sadeghi, V., 2014. J. Police Med. 3 (2), 78–84.
Ziegler, C., 2000. Fresenius' J. Anal. Chem. 366 (6–7), 552–559.