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Amperometric Immunosensor for the Detection of *Vibrio cholerae* O1 Using Disposable Screen-printed Electrodes

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A disposable amperometric immunosensor was studied for the rapid detection of *Vibrio cholerae* (*V. cholerae*), the causative agent of cholera, employing an indirect sandwich enzyme linked immunosorbent assay (ELISA) principle. Screen-printed electrodes (SPEs) were fabricated (by using commercial and homemade carbon inks), electrochemically characterized and the assay conditions were optimized for capturing antibodies and antigen. Whole cell lysate (WCL) of *V. cholerae* was used to raise antibodies in rabbits and mice. The antibodies raised against WCL of *V. cholerae* were found to be specific, and no cross reactivity was observed with other enteric bacteria. 1-Naphthyl phosphate was used as a substrate with the amperometric detection of its enzymatic hydrolysis product 1-naphthol at a potential of +400 mV *vs*. Ag/AgCl reference electrode. A comparison between the amperometric detection technique and the standard ELISA was made in terms of the total assay time, the amount of biological materials used and the sensitivity of detection. The minimum detection limit of the amperometric immunosensor for *V. cholerae* was found to be 10^5 cells/ml in 55 min, while ELISA detected 10^6 cells/ml in 4 h.

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Introduction

Vibrio cholerae (*V. cholerae*) causes the most severe epidemic gastrointestinal disease, cholera, which continues to be a health problem in countries with poor socio-economic conditions.[1] Almost every developing country is now facing either a cholera outbreak or the threat of an epidemic, which not only poses serious public health problems, but also affects the economy. The disease manifests with profuse watery diarrhea, vomiting and dehydration, often associated with leg cramps due to electrolyte imbalance, leading to severe dehydration and death. A large number of epidemics have been reported in various parts of India in recent years. Its environmental detection by active surveillance is very helpful because the signaling of a potential cholera outbreak could be a valuable tool for implementing preventive measures.[2]

Monitoring of the bacterium in water sources is traditionally performed by a series of biochemical tests.[3,4] In addition, several immunoassay procedures employed for the detection of *V. cholerae* are: polymerase chain reaction (PCR),[5-8] enzyme linked immunosorbent assay (ELISA),[9] coaglutination,[10] immunofluorescence[11-13] and quartz crystal microbalance (QCM).[14] Most of these methods somehow require highly qualified personnel (PCR), long time (ELISA) and sophisticated instrumentation (QCM, PCR). No reports are available for *V. cholerae* detection based on electrochemical techniques.

Over the past decade, interest has been increasing in concerning the development of a simple, inexpensive and disposable biosensor for the detection of pathogens under field conditions. Recently, there has been growing interest in using electrochemical immunosensors for environmental[15] and clinical diagnosis.[16-18] Electrochemical immunosensors combine the high specificity of traditional immunochemical methods with the low detection limit of a modern electrochemical system. The electrochemical techniques usually employed for immunoassays are mostly based on amperometric methods[16-19] and voltammetric methods.[20-22] For immunosensors based on electrochemical methods, it is also possible to miniaturize the electrode for field applications.[19] Wide varieties of electrodes such as the carbon paste electrode (CPE),[17,18,25,26] glassy carbon electrode,[20] platinum electrode[27] and gold electrode,[28] have been used for electrochemical immunosensors. Recently, several immunosensor devices have been developed on screen-printed electrodes (SPEs).[15,23,24] The advantages of screen-printing technology are that they are inexpensive and can be mass-produced.[28,29] Carbon inks are particularly attractive because they are relatively inexpensive and lead to low background currents and broad potential windows.[17] Various papers have been published on the fabrication of SPEs and their application for immunosensing.[30-33]

In our earlier studies, SPEs fabricated from Gwent carbon ink were found to be best, followed by PSG carbon ink for immunosensing purpose.[34] Therefore, these SPEs were applied for the present investigation. In the present study, we used rabbit anti-*Vibrio cholerae* IgG (as capturing antibodies) for the detection of *V. cholerae* (antigen) based on an indirect sandwich ELISA system. The capturing antibodies were adsorbed (physical adsorption) on SPE, which was subjected to sequential incubation in *V. cholerae* (antigen) and mice serum as revealing antibody and rabbit anti-mouse ALP conjugate. To the best of our knowledge, this is probably first report concerning *V. cholerae* detection by an amperometric immunosensor by using SPEs. In addition, we compared the conventional method (plate ELISA method) for the detection of *V. cholerae* with amperometric immunosensors using the same biological material for both methods.

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Experimental

Apparatus

Voltammetric and amperometric studies were performed with an Autolab PGSTAT 12 (Eco Chemie, The Netherlands) potentiostat/galvanostat and controlled by Autolab GPES software version 4.9 for Windows 98. The magnetic stirrer controller (Model No. TH100) supplied by Spectralab (India) was used to control the stirring speed. SPEs were prepared with a semi-automated screen-printer. The three-electrode assembly, consisting of a working electrode (electrode of interest *i.e.* SPE), a Ag/AgCl reference electrode and a platinum rod as a counter electrode was used for amperometric experiments. An ELISA plate reader (Biotech, USA) was used for plate ELISA experiments.

Reagents and solutions

Gwent carbon ink C1090D14 was purchased from Gwent Electronic Materials, Gwent, UK. Polystyrene was obtained from local sources. Eltecks (Bangalore, India) supplied graphite powder, and the dielectric ink. 1-Naphthyl phosphate, purified grade was obtained from Lancaster, UK. 1-Naphthol was obtained from SD-fine Chemicals (India). Tris(hydroxymethyl)aminomethane (Tris) buffer was purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) fraction V was obtained from John Baker Inc. (USA). $MgCl_2$ and NaCl were purchased from Sisco Chemicals (India). Rabbit anti-mouse immunoglobulin alkaline phosphatase conjugate (Dako code No. D0314) was received from Dako Cytomation, Denmark. Diethanolamine (DEA) and 4-nitrophenyl phosphate were purchased from Acros Organics.

A blocking buffer solution was prepared by dissolving 3% bovine serum albumin in Tris buffer (0.1 M; pH 7.2). Tris buffer pH 7.2 (for amperometric immunosensing) and PBS pH 7.4 (for plate ELISA) were used as washing buffers. A 0.1 M diethanolammine (DEA) buffer pH 9.8 (containing A 0.1 M of diethanolamine, 0.1 M sodium chloride and 1 mM of magnesium chloride) was used as an electrolyte in amperometric experiments. 1-Naphthyl phosphate and 1-naphthol solutions were made in a 0.1 M DEA buffer pH 9.8.

Clinical isolates of *V. cholerae* O1 were obtained form National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India. Other bacterial cultures used in this study *viz. Salmonella typhi, Escherichia coli* and *Shigella dysenteriae*, were available in the author's laboratory.

Procedures

Preparation of antigen

Whole-cell lysate of *V. cholerae* was prepared for use as an antigen in the production of antibodies. Briefly, *V. cholerae* O1 was grown in a brain heart infusion broth (BHI) overnight at 37˚C under shaking conditions. The bacterial cells were harvested by centrifugation at 10000*g* (Sigma Gmbh, Germany) for 10 min at 4˚C and washed thrice with saline (0.85% NaCl). The pellet was resuspended in saline and an aliquot was used for viable count determination. A suspension was heated at 95˚C for 15 min. The lysate was vortexed well and stored for use as an antigen. The sterility of the suspension was checked by streaking an aliquot on a BHI plate

Preparation of polyclonal antiserum

New Zealand white rabbit was initially injected intramuscularly with 10^7 cells of heat-killed *V. cholerae* with Freund complete adjuvant (Becton Dickinson, USA). Subsequently, three more doses were given with incomplete Freund adjuvant at an interval of 2 weeks. One week after the last injection, the animal was test bled from the marginal ear vein. Likewise, serum was raised in mice. However, the route of injection in mice was intraperitoneal. Serum titer was determined by plate ELISA using heat-killed *V. cholerae* O1 cells. Sera were kept in aliquots under frozen conditions.

Cross reactivity of antiserum

The cross reactivity of antiserum was tested against *E. coli*, *S. dysenteriae* and *S. typhi*. Heat-killed cells of the respective bacterium were suspended in carbonate buffer (pH 9.6) and 100 µl of samples containing 10^7 cells were added per well of a Maxisorb plate (Nunc). After overnight incubation at 4˚C, the wells were washed with phosphate buffer saline (PBS, pH 7.4) and blocked with 3% BSA in PBS overnight at 4˚C. After washing with PBS, 100 µl of anti-Vibrio serum dilutions made in 3% BSA in PBS was added and incubated for 1 h at 37˚C. After washing with PBS, 100 µl of anti mouse-ALP conjugate was added in each well (1:1000 in PBS). The enzymatic reaction was developed using 4-nitrophenyl phosphate in DEA buffer as the substrate after incubation for 1 h. The reaction was terminated after 15 min using 100 mM EDTA, and the absorbance values were recorded at 405 nm using an ELISA plate reader.

IgG purification

Two milliliters of rabbit serum were centrifuged at 2000*g* for 30 min at 4˚C, and 2 ml of 100% saturated ammonium sulfate solution was added to the supernatant with continuous stirring to precipitate serum proteins. Precipitated serum was centrifuged at 2000*g* after storing at 4˚C for 6 to 8 h, and the pellet was dissolved in PBS and used for immunoglobulin purification. A column (1.5 × 50 cm) was packed with a slurry of Biogel A-0.5 (agarose, exclusion limit of 500 kD) and equilibrated with sodium-phosphate buffer. One milliliter of precipitated serum sample was loaded on the column of a protein purification system (Biorad, USA). Fractions containing peaks were pooled and concentrated using a 30 kD filter membrane (Millipore, USA).

Fabrication of amperometric immunosensors (SPEs)

Homemade carbon ink (PSG) was prepared by mixing polystyrene dissolved in mesitylene and graphite particles. Ink containing 60% graphite and 40% polystyrene was found to be the optimized composition. A semi-automatic screen-printing machine was used to fabricate the SPEs. To fabricate SPEs (immunosensors), initially a layer of silver ink was screen-printed on the alumina substrate, and then carbon ink was screen-printed over the silver ink. Finally, a layer of insulation ink was screen printed over the electrode to expose an area of 7 mm^2. After screen-printing, the electrodes were dried at 80˚C for 2 h in an oven. The size of the electrodes was 37 mm × 5 mm.

Measurement procedure for amperometric immunosensor

First of all, an immunosensor was incubated with 5 µl of optimized dilution of the capturing antibody for 1 h, followed by sequential incubation with 5 µl of a solution containing *V. cholerae* cells, revealing antibody and optimized dilution of a rabbit anti-mouse ALP conjugate for 15 min each at 37˚C.

All amperometric experiments were performed at room temperature (27 ± 1˚C) in an electrochemical cell containing 10

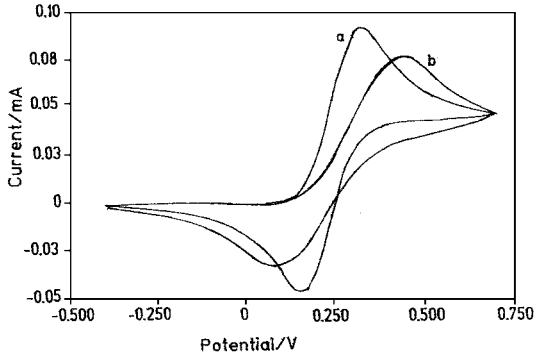

Fig. 1 Cyclic voltammogram for 1 mM ferrocyanide at (a) Gwent and (b) PSG screen-printed electrodes. A 0.1 M KCl was used as a supporting electrolyte. The scan rate was 20 mV/s.

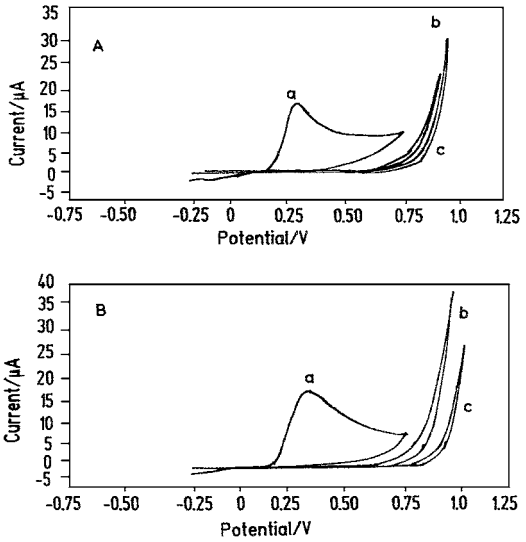

Fig. 2 Cyclic voltammogram at (A) Gwent and (B) PSG screen-printed electrodes in DEA buffer pH 9.8 for (a) 1-naphthol (b) 1-naphthyl phosphate and (c) buffer alone (blank). Scan rate, 20 mV/s.

ml of DEA buffer, pH 9.8 stirred at a constant rate of 700 rpm, as optimized in our earlier studies.[35] A potential of +400 mV *vs.* a Ag/AgCl reference electrode was applied. After reaching a steady state of the background current, the response was recorded by adding 1-naphthyl phosphate to a final concentration of 5 mM (electrochemical substrate for ALP enzyme), reported in our earlier studies.[34]

Measurement procedure for plate ELISA

A 10 µg/ml solution of purified IgG was made in carbonate buffer (pH 9.6), and 100 µl of this suspension was coated per well of a Maxisorb plate (Nunc). After overnight incubation at 4˚C, wells were washed with PBS, pH 7.4 and blocked with 3% BSA in PBS overnight at 4˚C. After washing with PBS, 100 µl of *V. cholerae* dilutions (containing 10^3 – 10^8 cells/ml) made in 3% BSA in PBS was added and incubated for 1 h at 37˚C. After washing with PBS, 100 µl of anti mice serum was added and incubated for 1 h. After washing with PBS, anti-mouse ALP conjugate (Dako) was added to each well (1:1000). The enzymatic reaction was developed using 4-nitrophenyl phosphate as the substrate after incubation for 1 h. The absorbance values were recorded at 405 nm using an ELISA plate reader (Biotech, USA).

Results and Discussion

Electrochemical characterization of immunosensors

A ferrocyanide redox system was used to study the voltammetric behavior of SPEs. Figure 1 displays cyclic voltammograms for ferrocyanide at Gwent and PSG SPEs. Gwent showed the most reversible behavior, lower oxidation potential and higher current than the PSG SPEs.

Figure 2 shows cyclic voltammograms for the substrate 1-naphthyl phosphate (0.2 mM), 1-naphthol (0.2 mM) and 0.1 M DEA buffer pH 9.8 alone (blank). The substrate 1-naphthyl phosphate and buffer alone (blank) shows no peak in the potential range from –200 to +700 mV, whereas 1-naphthol shows a characteristic peak at a potential of +300 mV and +350 mV *vs.* Ag/AgCl reference electrode, at Gwent and PSG SPEs, respectively. Thus, 1-naphthol, which is an enzymatic hydrolysis product of 1-naphthyl phosphate, could easily be detected by its electrochemical oxidation without any interference from the parent substrate (1-naphthyl phosphate) and the buffer (blank). Therefore, in amperometric experiments a potential of +400 mV was applied.

Optimization of parameters of assay procedure

The response of an immunosensor depends on several parameters of the assay procedure, like the optimized amount of the capturing antibody and the incubation time of *V. cholerae* cells. The number of *V. cholerae* cells captured on the surface of the immunosensor would be influenced by the amount of capturing antibodies already present on the immunosensor. Based on our earlier studies,[31] incubation times of 15 min and 1 h were fixed for anti-mouse ALP conjugate and for capturing antibodies, respectively.

For optimization of the anti-mouse ALP conjugate, first of all the SPEs were blocked with 3% BSA for 15 min at 37˚C. The amperometric response was measured after the addition of 1-naphthyl phosphate to a final concentration of 5 mM in the electrolyte. Then, the blocked SPEs were incubated with various dilutions of anti-mouse ALP conjugate. After each step, thorough washing was done with 0.1 M Tris buffer pH 7.2. A similarly amperometric response was measured for different dilutions of ALP conjugate; the dilution showing the response as that shown by 3% BSA blocked SPE was selected for further studies, as shown in Fig. 3. The optimized dilutions of anti-mouse ALP conjugate for PSG and Gwent SPEs are 1:250 and 1:100, respectively.

For optimizing the amount of capturing antibody, bare SPEs were incubated with various amounts of capturing antibody for 1 h, followed by blocking with 3% BSA for 15 min. Afterwards 1:500 dilution of mice serum (revealing antibody) was incubated for 15 min, and finally the optimized dilution of anti-mouse ALP conjugate was suspended for 15 min. All incubation steps were performed at 37˚C, and thorough washing was done with 0.1 M Tris buffer pH 7.2 after each step. Figure 4 shows that the response current for 10^7 cells of *V. cholerae* increased with increasing the amount of capturing antibody, and then became constant. Thus, an antibody of 0.02 µg/ml and 0.04 µg/ml for PSG and Gwent SPE was optimized for further studies. It was found that PSG SPE captured a greater amount of the capturing antibody, but gave the same response as that of Gwent SPE.

Measurement with amperometric immunosensor (SPEs)

Figure 5 depicts the steps involved in immunosensing. The

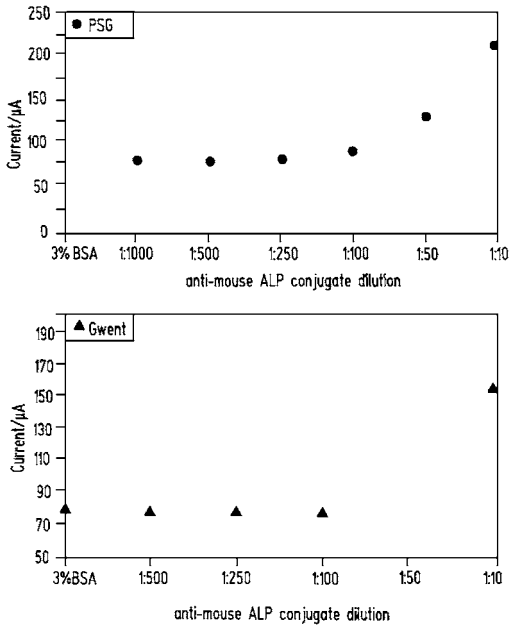

Fig. 3 Variation in the amperometric current with different dilutions of anti-mouse ALP conjugate in DEA buffer pH 9.8, at a potential of +400 mV *vs*. Ag/AgCl reference electrode with a stirring speed of 700 rpm.

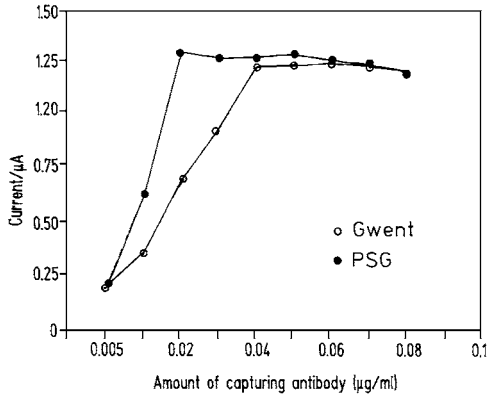

Fig. 4 Variation in the amperometric current *vs*. amount of capturing antibody on the SPE incubated for 1 h in a solution of 10^7 cells/ml of *V. cholerae*, followed by the same steps and conditions followed in the measurement procedure. ●, PSG; ○, Gwent.

amperometric detection of *V. cholerae* cells was done under optimized conditions. Figure 6 depicts the amperometric response of PSG and Gwent SPEs for various concentrations of *V. cholerae* cells. The detection limit of PSG and Gwent immunosensors for the *V. cholerae* cell was found to be 10^5 cells/mL, which corresponds to three-times the standard deviation of a blank (base line current) added to the blank response (0.50 μA and 0.51 μA for PSG and Gwent, respectively). The total analysis took 55 min for each experiment. This time includes the incubation time for the antigen, revealing antibody and anti-mouse ALP conjugate with intermittent washing and the time for the amperometric experiment. The relative standard deviation for Gwent and PSG immunosensors was found to be 4.4 – 4.9% and 6.1 – 6.8%, respectively (n = 5). Figure 7 shows the typical amperometric response curves (current *vs*. time plots for blank and various

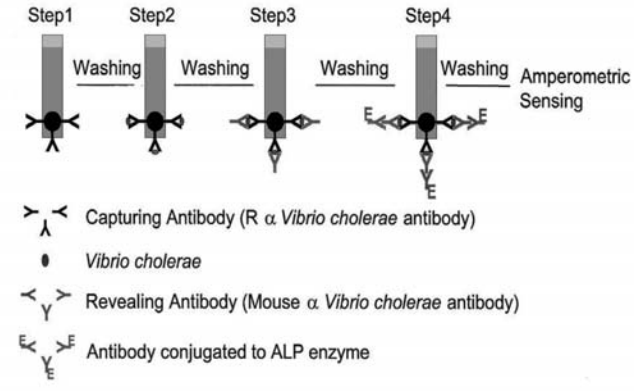

Fig. 5 Steps involved in immunosensing.

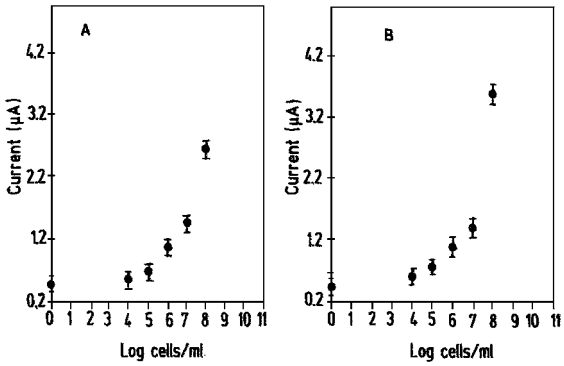

Fig. 6 Variation of the amperometric current with the concentration of *V. cholerae* cells under the optimized conditions at (A) PSG and (B) Gwent SPEs. The error bars represent the SD, 4.4 – 4.9% and 6.1 – 6.8% for Gwent and PSG SPEs, respectively (n = 5).

concentrations of *V. cholerae*) at PSG SPEs. The immunosensors were tested for the cross reactivity with 10^7 cells/ml of *E. coli*, *S. dysenteriae* and *S. typhi*; a small cross reactivity was found. Finally, the response current obtained for PSG and Gwent immunosensor was within *ca*. 0.50 ± 0.036 μA and *ca*. 0.51 ± 0.023 μA, respectively, and was similar to the response obtained for the blank.

Measurement with plate ELISA

Polyclonal antibodies raised against WCL showed a high titer in plate ELISA, when heat-killed *V. cholerae* O1 cells were used as an antigen. Lower dilutions of serum reacted with other enteric bacteria *viz. E. coli*, *S. dysenteriae* and *S. typhi*, but the absorbance values were very low *i.e*. negligible values at lower dilutions (Fig. 8). To determine the sensitivity of the plate ELISA detection system, *V. cholerae* O1 cells were serially diluted 10 fold in PBS. The detection limit for the *V. cholerae* cell was found to be 10^6 cells/ml.

Conclusions

The indirect sandwich ELISA principle was used with ALP-labeled antibodies being used in amperometric detection. Amperometric detection method was chosen owing to many benefits, including its inherent ability used in miniaturized, portable systems. Disposable SPEs, the main element of this detection system, were fabricated from carbon inks. In our

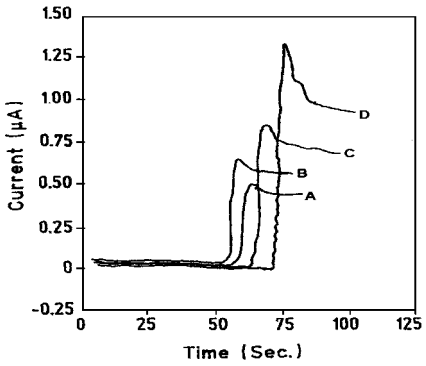

Fig. 7 Typical amperometric response curves for the blank (A), 10^5 cells/ml (B), 10^6 cells/ml (C) and 10^7cells /ml (D) of *V. cholerae* cells at PSG SPEs in DEA buffer pH 9.8. Applied potential +400 mV *vs.* Ag/AgCl reference electrode with the stirring speed of 700 rpm.

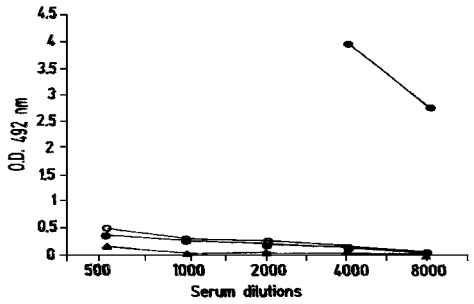

Fig. 8 Reactivity of polyclonal antiserum raised against WCL of *V. cholerae* O1 with *E. coli* (△), *S. dysenteriae* (●), *S. Typhi* (○) and *V. cholerae* O1 (◆).

earlier studies, for the detection of MIgG by using Gwent SPE, the sensitivity was two-times better than that of PSG SPE. However, it is interesting to know that both types of SPEs showed similar sensitivity towards *V. cholerae* detection, though Gwent showed a lower relative standard deviation than the PSG immunosensor. The data showed that 10^5 cells/ml of *V. cholerae* could be detected in 55 min and using a much smaller volume (5 μl) of immunological reagents. However, the plate ELISA method showed a detection limit of 10^6 cells/ml; the required time was 4 h and 100 μl of immunological reagents was used. Finally, it can be concluded that the amperometric immunosensing is faster and more sensitive than the well-known plate ELISA method.

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