VARIABILITY IN AN ENZYME-LINKED, IMMUNOSORBENT ASSAY (ELISA) FOR ERWINIA CAROTOVORA SUBSP. ATROSEPTICA

by

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Factors affecting a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of Erwinia carotovora subsp. atroseptica were investigated. Optimum reaction conditions for detecting known cell numbers of Eca were found to be 2.0 \mu g/ml of coating \gamma-globulin and 1:400 enzyme-\gamma-globulin conjugate dilution. These conditions were determined using antiserum produced against glutaraldehyde-fixed, whole bacterial cells of strain E82 of Eca (serogroup I), and polystyrene microtitration plates (Dynatech substrate plates). In spite of these optimized conditions, variability was observed between sets of data obtained under identical experimental conditions. In order to minimize or eliminate this variability, different parameters were investigated. The washing procedure was standardized by the use of a controlled pressure-washing system employing distilled water, and two 15-sec washes at 34.48 kPa (5 psi), with 180° rotation of the plate between each wash. Tween-20 was eliminated from the washing solution, since it interfered with the sensitivity of the assay. This effect could not be related to the age of the Tween-20 employed. Well to well variability was observed with the polystyrene microtitration plates employed but it was not exclusive to the outside rows. The pattern of distribution of the "odd" wells within a plate changed, and the number of "odd" wells decreased with time. The maximum variation from the mean also decreased with time. Addition to different wells of an extra 5% of coating \gamma-globulin, sample, and enzyme-\gamma-globulin conjugate individually or in different combinations, failed to reproduce the variability observed thereby eliminating pipetting errors as a source of variability. The \text{A}_{0.05} values were influenced by the buffer solutions employed for sample and conjugate dilution. Any given buffer had a greater effect when used for
conjugate dilution. The complete buffer of phosphate buffered saline (PBS)+
0.05% Tween-20 +2.0% polyvinylpyrrolidone+0.2% egg albumin commonly used in
virus work, was found to be suitable for the Eca system although its effi­
ciency in the presence of plant material containing bacteria remains to be
evaluated.

This ELISA for Eca employing optimized coating and conjugate, a stan­
dardized washing procedure and a complete buffer for samples and conjugate
dilution, routinely detected 10^5 to 10^6 cells/ml of only serogroup I of Eca
when pure cultures of both homologous and heterologous strains were tested.
At concentrations >10^7 cells/ml, strains from serogroups XVIII, XX, and XXII
of subsp. atroseptica and a few strains from serogroups II, III, IV, and V of
subsp. carotovora also reacted. Even at high bacterial concentration (10^8
cells/ml) no cross reactions were observed with Pseudomonas marginalis and
Corynebacterium sepedonicum. Heat treatment of cell suspensions of serogroup
I at 60 C for 3-6 min enhanced A_405 values but the level of sensitivity was
not reduced below 10^5 cells/ml. Cross reactions with strains of subsp. caro­
tovora serogroups III and V, observed at 10^7 cells/ml, were reduced but not
eliminated by this heat treatment. Both heat-labile and heat-stable water­
soluble antigens were detected by this ELISA for Eca. The media upon which
cells were grown also affected the A_405 values but this effect was not propor­
tional to the amount of growth observed.

Based on these results it was concluded that until well to well varia­
bility is eliminated, and sensitivity increased, there will be little incen­tive to use the double sandwich ELISA technique with plant sap where a reduc­
tion in sensitivity is likely. At this point ELISA seems to have little
potential in routine surveys for detecting latent blackleg infections in
certified seed potatoes.
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INTRODUCTION

Plant pathogenic bacteria, unlike fungi, cannot be identified on the basis of morphology. As a consequence, reliable and specific detection and identification techniques are required. Because many bacterial pathogens exist in low numbers in a latent state until triggered into activity by favorable environmental conditions, these detection techniques must also be very sensitive if they are to be of any practical use. Antibiotic-resistant strains and the lack of effective substitutes registered for use have resulted in generally poor chemical control. This in turn has resulted in certification becoming an important component in the strategy for bacterial disease control. However, the existence of latent infections makes certification on the basis of field symptoms difficult to justify. In the absence of reliable, specific and sensitive techniques there is no alternative. Until recently, such was the situation with Erwinia carotovora.

Erwinia carotovora is now thought to overwinter primarily in latent infections in the lenticels of potato tubers. The environmental conditions during storage and the growing season, determine when and if the tuber or seed piece will start to rot. Thus, storage rot, seed piece decay, field blackleg or tuber rot at harvest may or may not occur. Even though the seed piece breaks down and contaminates the daughter tuber and the rhizosphere, the tops may not show any visible symptoms. Thus, techniques to detect these latent infections which could be used to predict storage ability or for seed potato certification purposes are surely needed. Fortunately in the last few years, several different techniques have been developed so that the detection of Erwinia carotovora is further advanced than with most other plant pathogenic bacteria.
The use of selective and enrichment media is one means by which phytopathogenic bacteria can be detected. These media, designed to select for growth of a certain organism, can not always prevent the growth of unwanted organisms, and are generally of low sensitivity. For example, crystal violet pectate (CVP) (Cuppels and Kelman 1974) allows a minimum detection of $10^6 - 10^7$ cells/g dry wt soil of Erwinia carotovora, but permits growth of Pseudomonas spp. and other bacteria. If used in combination with Meneley and Stanghellini's enrichment medium, the level of sensitivity can be increased by 100 to 1,000-fold (Meneley and Stanghellini 1976).

The long standing capability of clinical medical microbiology to provide rapid and accurate serological identification of bacteria, would suggest a similar potential exists for plant pathogenic bacteria. Only recently have different techniques based on this principle been fully evaluated. The agglutination test has been employed to identify or differentiate numerous phytopathogenic bacteria including Erwinia atroseptica (Graham 1963), E. aroideae (Okabe and Goto 1955), E. carotovora (Goto and Okabe 1957), and E. amylovora (Lelliott 1968). The general experience with this technique is that high cell concentrations are required. While no figures appear to be available for Erwinia carotovora, Slack et al. (1979) have reported that Corynebacterium sepedonicum could be detected only at a level of $2 \times 10^7$ cells/ml by this technique. In the same study, the immunodiffusion test permitted a similar level of sensitivity. Vruggink and Maas Geesteranus (1975) detected $10^7$ cells/ml of Erwinia carotovora subsp. atroseptica (Van Hall) Dye (Eca) by immunodiffusion. However, the value of the immunodiffusion technique is in determining relationships (DeBoer et al. 1979), rather than in detection. The latex agglutination test represents another possibility that has not been evaluated for use with Erwinia carotovora, probably because of the early
application of immunofluorescence (IF) to detection of \textit{Eca} by Allan and Kelman (1977). Using direct IF they were able to detect $10^5 - 10^6$ cells/ml. The indirect technique (Slack \textit{et al.} 1979) has been reported to be even more sensitive ($10^1 - 10^2$ cells/ml) with \textit{Corynebacterium sepedonicum}. DeBoer (1980) has successfully used the direct technique to detect cross reaction between \textit{Erwinia carotovora} serogroups, on the basis of their flagellar antigen. However, the IF technique is not always easy to perform (Bar-Joseph \textit{et al.} 1979), is time consuming, and depends upon subjective assessment (Voller \textit{et al.} 1974, 1976, 1977).

The enzyme-linked immunosorbent assay (ELISA) which has found widespread application in medicine (bacterial, mycotic, viral and parasitic diseases), and veterinary science (viral, mycoplasmal, and parasitic diseases) (Voller \textit{et al.} 1979), is a serological technique that has become available to plant pathology only recently with the adaptation of the technique by Clark and Adams (1977) for plant viruses. It probably offers the greatest potential for pathogen detection in plant pathology (Clark 1981).

The basic ELISA test depends on two principles. The first, is that an antigen or antibody can be attached to a solid phase support yet retain immunological activity. The second, is that either antigen or antibody can be linked to an enzyme, and the complex retains both immunological and enzymatic activity (Voller \textit{et al.} 1976, 1977).

Different types of assays employing these basic principles have been described. The competitive method, the double antibody sandwich method, and the modified double antibody sandwich can all be used for detection and measurement of antigen. The inhibition ELISA for antigen detection is especially suited for use with small molecular weight substances. The
indirect method can be used for detection and measurement of antibodies and has found widespread application especially with viral diseases (Voller et al. 1979). The double antibody sandwich ELISA method is the most commonly employed with plant viruses, bacteria, and spiro plasma. It is carried out as follows: the specific antibody is adsorbed to a solid phase, and the excess is removed by washing. The test solution containing the antigen is added, and the excess is washed away after an appropriate incubation period. The enzyme labelled specific antibody is added, and the excess removed by washing. The enzyme substrate is added and the amount of substrate hydrolysis detectable as color development is proportional to the amount of antigen present (Voller et al. 1979).


When compared to the other serological techniques currently employed with different antigen systems, ELISA proved to have many advantages. Flegg and Clark (1979) reported that ELISA was more sensitive than the tube precipitin test when employed for Apple Chlorotic Leafspot Virus. Similarly, ELISA was better than the complement fixation test for detection of cytomegalovirus IgG antibody (Chia and Spence 1979). It was more sensitive than agglutination
and immunodiffusion for detection of Rhizobium (Kishinevsky and Bar-Joseph 1978), and a lot more sensitive \(10^3 - 10^5\) x than double diffusion for the detection of Phoma tracheiphila responsible for Mal Secco disease in lemon (Nachmias et al. 1979). Voller et al. (1974), in their work with malaria, found a positive correlation between ELISA and immunofluorescence, but also pointed out that the latter was more time consuming. Similarly, Bar-Joseph et al. (1979) reported that ELISA was easier to use than immunofluorescence with Citrus Tristeza Virus. ELISA was also found to have a comparable sensitivity to the Radio Immuno Assay (RIA) (Engvall et al. 1971; Engvall and Perlmann 1972; Voller et al. 1976, 1977; Yolken et al. 1977), while overcoming the subjectivity, expense, risks, and restricted use associated with RIA (Voller et al. 1976, 1977, 1979). Furthermore, the possibilities of the ELISA technique for large scale sampling (Bar-Joseph et al. 1979; Carlier et al. 1979; Chia and Spence 1979; Clark and Adams 1977; Clark et al. 1978; Flegg and Clark 1979; Kishinevsky and Bar-Joseph 1978; Marco and Cohen 1980; Stevens and Tsiantos 1979; Tamada and Harrison 1980; Tresh et al. 1977), make it an attractive technique for certification programs, where several virus and bacterial diseases could eventually be assayed at once.

In much of the preliminary work with plant-infecting bacteria, the technique has not shown the expected high level of sensitivity predicted from the success obtained in virus detection (Tresh et al. 1977; Clark and Adams 1977; Bar-Joseph et al. 1979). Kishinevsky and Bar-Joseph (1978) reported a level of detection of only \(10^6 - 10^8\) cells/ml for Rhizobium under normal conditions. Weaver and Guthrie (1978) considered \(10^6 - 10^7\) cells/ml a high level of sensitivity for Pseudomonas phaseolicola. Stevens and Tsiantos (1979) used ELISA to detect whole Corynebacterium michiganense cells, both in culture
suspensions of known concentrations and tomato plant extracts, and reported a level of detection of $10^3$ cells/ml from pure culture suspensions. However, this conclusion is not supported by their data, and a level of $10^5 - 10^6$ cells/ml seems more realistic. Claflin and Uyemoto (1978) reported detecting Corynebacterium sepedonicum in infected stems and tubers of potato, and in culture suspensions. Unfortunately, no level of sensitivity was reported. Cross reactions have also been a frequent problem in these preliminary reports. Berger et al. (1979) demonstrated weak cross reactions in ELISA with heterologous antisera for Rhizobium strains. Weaver and Guthrie (1978) reported cross reactions between Pseudomonas phaseolicola and other unspecified bacteria. Similarly, Vruggink (1978) reported cross reactions between Xanthomonas pelargonii and Aplanobacter populi.

The only instance where ELISA has been used with some success with phytopathogenic bacteria has been with Eca (Vruggink 1978; Cother and Vruggink 1980). However, while cautioning about the need for determining the limits and specificity of the assay, Vruggink did not provide experimental evidence that many of the factors affecting the assay with other organisms had been investigated for the Eca system. The detection of artificially-created latent infections of Eca in potato tubers (Cother and Vruggink 1980) indicated the potential usefulness of the ELISA technique.

The ELISA technique if it is to be widely used for bacteria must overcome the lack of sensitivity and specificity observed in the preliminary studies. These studies have largely employed reaction conditions used for plant virus assays. Whether these failures are due to the fact that bacterial and viral systems have different optimal reaction conditions needs to be determined. Identification of the sources of variability observed in other
systems (Bullock and Walls 1979; Carlsson et al. 1972, 1975; Engvall et al. 1971, and Lister and Rochow 1979) and means for their removal are essential to any attempt to increase sensitivity in bacterial systems. Of the phytopathological bacterial systems studied to date only that involving \textit{Eca} seems to offer much promise. In addition, considerably more is known about the serological relationships of this organism (cross reactions between serogroups, presence of flagellar and somatic antigens) and there is an immediate practical application for an ELISA for this organism. Thus, \textit{Eca} was selected as the model ELISA system for study with the following objectives:

1. to devise a standardized working system and determine the optimum working conditions;
2. to determine the optimum buffers for sample and conjugate preparation;
3. to evaluate the microtitration plates, pipetting errors and washing technique as sources of variability;
4. to determine the effect on sensitivity of heat treatment and washing of bacterial cells;
5. to determine whether the immunodiffusion serogroup specificity was retained in the ELISA.
CHAPTER I. EFFECT OF THE PLATE WASHING PROCEDURE ON THE DETECTION OF ERWINIA CAROTOVORA SUBSP. ATROSEPTICA (VAN HALL) DYE BY THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

INTRODUCTION

The enzyme-linked immunosorbant assay (ELISA) has found widespread application in medicine (bacterial, mycotic, viral and parasitic diseases), veterinary science (viral, mycoplasmal and parasitic diseases), and more recently agriculture (viral diseases) (Voller et al. 1979). However, as Lehtonen and Viljanen (1980) have pointed out it has been widely used without adequate standardization. To realize the full potential of ELISA, optimal conditions must be precisely determined for each system (Clark 1981; Voller et al. 1977). The need for standardization of reagents, operational procedures and methods for the analysis and presentation of results, as well as the need for a better appreciation of the principles involved becomes correspondingly greater as the technique becomes more widely used in plant pathology (Clark 1981). The operational procedure for each system requiring standardization should include determination of a suitable coating-conjugate combination, choice of buffer for preparation of sample and conjugation dilutions, time and temperature of incubation, enzyme system, reaction time for substrate and a convenient method of data analysis.

The procedure employed to wash the microtitration plates between steps has been described as the key to the successful use of this method (Birch et al. 1979; Voller et al. 1977). A survey of the literature indicated that not only has a variety of washing solutions been employed, e.g. tap water (Bidwell
et al. 1977; Henrikson 1979), distilled water (Bruins et al. 1978), and phosphate buffered saline containing 0.05% Tween-20 (PBS+T) (Berger et al. 1979; Birch et al. 1979; Brodeur et al. 1978; Carlsson et al. 1976; Clark and Adams 1977; Engvall and Perlmann 1971; Flegg and Clark 1979; Holmgren and Svennerholm 1973; Voller et al. 1979), but that the number of washes, and the washing time varied greatly. PBS+T is the most widely employed washing solution, presumably because the addition of Tween-20 was reported to increase sensitivity by Bullock and Walls (1977) working with Toxoplasma gondii. Bruins et al. (1978) have reported that its inclusion in the washing solution actually decreased sensitivity probably because the detergent action removed from the polystyrene walls the lipopolysaccharide of the rough mutant of Salmonella minnesota being studied. The washing procedure itself has rarely been described. Excepting the few instances where either an automatic washer (Denmark and Chessum 1978), or a homemade washing device (Henriksen 1979) was employed, the use of a wash bottle must be assumed.

Although ELISA has been employed with several genera of plant pathogenic bacteria including Corynebacterium michiganense (E. F. Sm) Jensen (Stevens and Tsiantos 1979), Corynebacterium sepedonicum (Spieck. & Kotth.) Skapt. & Burkh. (Claffin and Uyemoto 1978), Pseudomonas phaseolicola (Burkh.) Dowson (Weaver and Guthrie 1978) and Xanthomonas pelargonii (N.A. Brown) Starr & Burkh. (Vruggink 1978), no data on the determination of optimal conditions were presented for these systems. Vruggink (1978) and Cother and Vruggink (1980), working with Erwinia carotovora subsp. atroseptica (van Hall) Dye (Eca) cautioned about the need for determining the limits and specificity of the assay, but did not provide experimental evidence that many of the factors affecting ELISA in other systems had been investigated in the Erwinia system.
However, their work did indicate that the technique had potential with this organism.

The objectives of this work were to determine optimal reaction conditions for the detection of *E. carotovora* subsp. *atroseptica* (van Hall) Dye by ELISA and to determine the effects of different washing procedures, and washing solutions on a standardized model system.
MATERIALS AND METHODS

Bacterial Cultures, Antiserum Production, and γ-globulin Purification

Potato strains (E82, E193) of *Eca* conforming to serogroup I (DeBoer et al. 1979) were employed throughout this study. Stock cultures were maintained on Difco Nutrient Agar (NA) slants at 4 C. Unless otherwise noted, bacterial cell suspensions used in the assays were prepared from 48-hour cultures grown on NA at 27 C. Antiserum against glutaraldehyde-fixed, whole cells of strain E82 was prepared in rabbit by the procedure of Allen and Kelman (1977).

To avoid excessive loss of γ-globulin by adsorption to glass surfaces, all glassware employed in the purification procedure was siliconized with a 5% solution of dichlorodimethyl silane \((\text{CH}_3)_2\text{SiCl}_2\) (Matheson, Coleman and Bell Manufacturing Chemists) in chloroform. The γ-globulin purification procedure of Clark and Adams (1977) was followed, with some modifications. One ml of crude antiserum was diluted in 9.0 ml of sterile distilled water, and the γ-globulin precipitated by dropwise addition of 10 ml of saturated ammonium sulfate. After stirring for 60 min at room temperature, the resulting suspension was centrifuged at 2500 g for 5 min at 4 C (IEC Refrigerated Centrifuge, Model B-20, Rotor 870). The pellet was resuspended in 1.0 ml of half strength phosphate buffered saline (PBS), 0.005 M, pH 7.4. The dilution, precipitation and centrifugation steps were repeated and the pellet obtained resuspended in 1.0 ml of half-strength PBS, and dialyzed at 4 C against three changes (two, 1-hour periods and overnight) of 500 ml of half-strength PBS. The dialyzate was passed through a DEAE-22 Sephadex column (3.0 - 5.0 x .9 cm) preequilibrated by passing 10x, 5x, 1x, and half-strength PBS, pH 7.4 through a batch of DEAE-22 until pH and conductivity matched half-strength PBS. The γ-globulin
was washed through with half-strength PBS and the effluent monitored at 280 nm with an ISCO Model UA-4 Absorbance Monitor. The first major peak was collected and the combined fractions were adjusted to $A_{0.05} = 1.4$ (approximately 1 mg of $\gamma$-globulin/ml) using a Gilford Spectrophotometer 250. The $\gamma$-globulin was divided into 0.5 ml aliquots in siliconized 15 x 45 mm, screw-cap glass specimen bottles, and stored at -18 C.

Conjugation of Alkaline Phosphatase with $\gamma$-globulin.

The conjugation procedure of Clark and Adams (1977) was followed. Siliconized glassware was used in all experiments. A 0.5 ml aliquot of alkaline phosphatase (Phosphatase Alkaline No P-4502, Sigma Chemical Co.) was centrifuged at 2500 g for 5 min at 4 C (IEC Rotor 870). The pellet was resuspended in 1.0 ml of purified $\gamma$-globulin and dialyzed at 4 C against three changes (two, 1-hour periods, and overnight) of 0.01 M, pH 7.4 PBS (500 ml). Glutaraldehyde was added directly to the suspension to give a final concentration of 0.06%, and the mixture incubated 4 h in the dark at room temperature. The excess glutaraldehyde was removed by dialysis as previously described. The alkaline phosphatase-$\gamma$-globulin conjugate was stored in siliconized vials at 4 C and approximately 0.0001% of sodium azide was added as a preservative.

ELISA

The ELISA procedure described by Clark and Adams (1977) was employed with modifications as noted for individual experiments. Flat bottom polystyrene microtitration plates were employed (MicrotiterR Immulon Substrate Plates, Dynatech Laboratories Inc., Alexandria, Virginia). Prior to coating with $\gamma$-globulin all plates required for a given experiment were numbered, and scanned at 405 nm on a Titertek Multiskan plate reader (Flow Laboratories,
Model 310C). Column one of plate one was employed as a blank to calibrate the instrument. Only plates with uniform $A_{405}$ values were used. The coating $\gamma$-globulin, prepared in carbonate buffer, pH 9.6 (0.15% NaCO$_3$ + 0.29% NaHCO$_3$ + 0.026% NaN$_3$), was added (0.2 ml) to each well. The plates were placed individually in plastic bags and incubated at 37 C for 4 h.

A plate washing device (Figure 1) which permitted control of the duration and pressure of the wash solution was used to standardize the washing conditions. Plates to be washed were emptied of contents, shaken vigorously to remove droplets trapped in the wells, and placed inverted on the "rinsing box". Following a 15-sec wash with distilled water (DW) at 34.48 kPa (5 psi) the plates were removed, shaken to remove droplets, rotated 180 degrees and similarly washed a second time. Plates were removed, shaken and placed on a similar box connected to compressed air (Linde Medical Air, Breathing Grade, CGA Type 1, Grade F). After a 5-sec drying period at 172.38 kPa (25 psi), the plates were removed, rotated 180 degrees, and the treatment repeated.

Bacterial cells grown as previously described, were suspended in a fresh solution of phosphate buffered saline containing 0.05% Tween-20 (polyoxyethylene (20) sorbitan monolaurate) (Fisher Scientific), 2.0% polyvinylpyrrolidone (PVP) (M.W. approx. 44,000, BDH Chemicals), and 0.2% egg albumin (EA) (Egg albumine (ovalbumine) Grade III, Sigma, No-5378), hereafter referred to as PSB+T+PVP+EA. The bacterial suspension was adjusted to an absorbance at 540 nm ($A_{540}$) of 0.1, which corresponded to $10^8$ cells/ml. A ten-fold dilution series was prepared in the same sample buffer. Buffer alone with no bacterial cells added, served as the control. Aliquots (0.2 ml) of the appropriate bacterial concentration were added to the wells. The plates were placed individually in plastic bags, incubated at 4 C overnight (16 hours) and were
Figure 1 - Description of the controlled-pressure washing system. The controlled pressure washing system consisted of a time clock (2) linked to both the pump (3), and the solenoid valve (6) controlling liquid movement. The pressure was adjusted with the pressure regulator (4), and monitored on the pressure gauge (5). The pump was linked to a reservoir (1) containing the appropriate washing solution. The "rinsing box" (7) was composed of a plexiglass frame, supporting an inverted microtitration plate, each well of which had been replaced by a 1 cc tuberculin syringe cover provided with a 45° angle needle hole at their tip (designed by Dr. R. Stace-Smith, Vancouver Research Station, Agriculture Canada). (8) Microtitration plate to be washed. (a) 2.0 cm glass-fiber reinforced tygon tubing (b) 1.5 cm copper pipe. (c) 1.0 cm tygon tubing).
washed as previously described. The alkaline phosphatase-γ-globulin conjugate was also prepared in a fresh solution of PBS+T+PVP+EA and 0.2 ml added to each well. The plates were placed individually in plastic bags, and incubated at 37°C for 4 h prior to washing as previously described. Aliquots of 0.2 ml of alkaline phosphatase substrate (p-nitrophenyl phosphate disodium, crystalline, Sigma, Sigma 104) at 0.6 mg/ml in a 10% diethanolamine solution, pH 9.8, were added to the plates, with the use of an eight channel Multi Channel Pipetter (Titertek), calibrated to deliver 0.2 ml. The reactions were allowed to proceed for 30 min at room temperature. A qualitative visual estimate of the degree of yellow color development was made, and the plates were read spectrophotometrically at 405 nm with a Titertek Multiskan plate reader calibrated against the first column wells at the start of the reaction.

\[ \frac{A_{405 \text{ sample}}}{A_{405 \text{ control}}} \] 

An absorbance ratio \( \frac{A_{405 \text{ sample}}}{A_{405 \text{ control}}} \) greater than 2.0 was considered a positive result.

**Determination of the Optimum Coating γ-globulin Concentration and Enzyme-γ-globulin Conjugate Dilution**

Coating γ-globulin concentrations of 4.0 μg, 2.0 μg, 1.0 μg, and 0.5 μg/ml were prepared; a treatment with coating buffer alone served as the control. Alkaline phosphatase-γ-globulin conjugate dilutions of 1:100, 1:200, 1:400, and 1:800 were prepared; conjugate buffer alone served as the control. A ten-fold dilution series of bacteria ranging from \( 10^3 \) to \( 10^7 \) cells/ml was employed; buffer alone with no bacterial cells added served as the control. Two microtitration plates were required to contain all possible coating-
sample-conjugate combinations. Three replicates were employed. On each plate, wells of columns 1, 12, and Row A from column 1 to 6 received only the buffers, and the substrate.

**Kinetics of the Reaction**

Bacterial dilution series ranging from $10^3$ to $10^8$ cells/ml of strain E193 and E82 were prepared. Each dilution of each strain was replicated six times on one microtitration plate (84 wells), and compared to the sample buffer control. The reaction was assessed immediately after addition of the substrate and every 5 min thereafter.

**Washing Procedure Standardization**

In all experiments with the washing procedure, a coating $\gamma$-globulin concentration of 2.0 $\mu$g/ml, conjugate dilution of 1:400, bacterial concentrations of $10^3$ to $10^7$ cells/ml, and a buffer only control were employed. Each bacterial concentration including the control represented a treatment and was replicated 10 times/plate. The outside rows of each plate contained only the buffer control and the substrate. The ELISA procedure was performed as previously described, except that the washing procedure was modified. The washing solutions evaluated were phosphate buffered saline (PBS), PBS containing 0.05% Tween-20 (PBS+T), and distilled water (DW). In initial experiments the use of a wash bottle was compared with the controlled-pressure washing system. One microtitration plate was employed/buffer/system. One wash bottle was used for each buffer and the microtitration plates were washed three times for 1 min at each washing step. A compressed air drying period was not employed with this conventional washing technique, but plates were shaken to remove droplets after each washing step. With the controlled-pressure wash-
ing system, the washing solutions were kept in individual containers, and the system flushed with the washing solution prior to use to remove the previous washing solution. To facilitate the procedure, the buffers were employed in the following order: DW, PBS, and PBS+T. To eliminate any trace of Tween-20 the system was flushed with DW for 2 to 3 min at the completion of each PBS+T wash. The plates were washed twice for 10 sec at 34.48 kPa (5 psi), and dried with compressed air twice for 5 sec at 172.38 kPa (25 psi).

The effect of air drying the plates with compressed air, was evaluated in only the controlled pressure washing system, employing two washes of 10 sec at 34.48 kPa (5 psi). At each wash step, one series of plates were only shaken, while a second series were shaken and dried with compressed air previously described.

A comparison was also made between different lots of Tween-20 (Fisher Scientific) used for the preparation of PBS+T. The first two Tween-20 lots (T1, T2) were approximately one year old, T1 representing the lot previously used; the third lot (T3) was recently purchased and had 6 months left before the expiration date. The plates were washed twice for 10 sec at 5 psi, and dried twice for 5 sec at 172.38 kPa (25 psi) at each wash step.

Up to this point, all experiments involving the controlled pressure washing system, were conducted with an arbitrary choice of conditions in the duration, number of washes, and pressure. To optimize the washing procedure, an experiment was designed with the following variables: one, two, or three washes at each step, for periods of 5, 10, 15, or 20 sec, and a pressure of 34.48 kPa (5 psi) or 68.95 kPa (10 psi). Only DW was employed, and two drying periods of 5 sec at 172.38 kPa (25 psi) applied at each wash step.
To determine the effect of washing on a system employing only a protein antigen, similar experiments were conducted with a purified preparation of a strain of Dandelion Virus S (DVS) obtained from L. Johns (Canada Department of Agriculture, Vancouver Research Station). Previous work (L. Johns personal communication) had established that 1.0 μg/ml of coating γ-globulin and a conjugate dilution of 1:800 were optimal. A ten-fold dilution series was prepared from a stock DVS preparation containing 140 ng/ml. The conventional washing procedure utilizing a wash bottle with PBS+T, (three washes of 1 min at each wash step without drying) was compared with the standardized washing conditions of two washes of 15 sec at 34.48 kPa (5 psi) with DW, followed by two drying periods of 5 sec at 172.38 kPa (25 psi), at each step. One microtitration plate was employed for each system. Wells of columns 1, 12, Row A from column 1 to 5, and Row H from column 8 to 12 of each plate, contained only the buffer control and the substrate.

Statistical Analysis

In all experiments, all treatments were completely randomized. In some experiments, Tukey's multiple range test was performed on \( A_{405} \) values uncorrected for their controls with a 5.0% significance level for the F-value.
RESULTS

Determination of the Optimum Coating \( \gamma \)-globulin Concentration and Enzyme-\( \gamma \)-globulin Conjugate Dilution

At bacterial cell concentrations of \( 10^5 - 10^7 \) cells/ml, color development (\( A_{405} \)) in this ELISA for \( Eca \) decreased as the dilution of enzyme-\( \gamma \)-globulin conjugate was increased (Fig. 2). At \( 10^7 \) and \( 10^6 \) bacterial cells/ml, the mean values obtained for each conjugate dilution within each coating concentration were significantly different from one another. However with \( 10^5 \) cells/ml, this pattern was not maintained. At this concentration and at 4.0 \( \mu \)g/ml all conjugate dilutions were significantly different from each other except 1:200 and 1:400. At 2.0 \( \mu \)g/ml 1:100 and 1:200, and 1:200 and 1:400 did not differ, but 1:100 was significantly different from 1:400. The highest dilution (1:800) was significantly lower than all of the others. Only the differences between 1:100 and 1:400, 1:100 and 1:800, and 1:200 and 1:800 were significantly different at 1.0 \( \mu \)g/ml. At 0.5 \( \mu \)g/ml only 1:100 and 1:800 differed significantly.

Color development was also a function of bacterial concentration and coating \( \gamma \)-globulin concentration. Most of the mean \( A_{405} \) values among the coating concentrations within the same conjugate dilution at \( 10^5 \), \( 10^6 \) and \( 10^7 \) cells/ml were significantly different. Exceptions were at \( 10^7 \) cells/ml with 1.0 and 2.0 \( \mu \)g/ml coating \( \gamma \)-globulin with both 1:100 and 1:200 conjugate dilutions and with 2.0 and 4.0 \( \mu \)g/ml coating \( \gamma \)-globulin and 1:800 conjugate dilution.

Further exceptions were at \( 10^5 \) cells/ml at 1:400 and 1:800 conjugate dilutions were there was not a significant difference between the 2.0 \( \mu \)g/ml...
Fig. 2. Effect of coating γ-globulin concentration and enzyme-γ-globulin conjugate dilution (1:100 , 1:200 , 1:400 , 1:800 ) on absorbance (A$_{0.05}$) in an ELISA for Erwinia carotovora subsp. atroseptica at known cell concentrations. Each value is the mean of three replicates.
and 4.0 μg/ml coatings, and at 1:800 where the difference between the 1.0 and 2.0 μg/ml coatings was also not significant.

The mean control values ($A_{405}$) also decreased with a decrease in coating concentration and an increase in conjugate dilution (Fig. 2 and Table 1). Because the background was a function of the coating-conjugate combination, the high coating and conjugate combinations rapidly gave visually positive backgrounds. Those combinations which developed positive background readings within 30 min were not further considered. When the data in Fig. 1 were examined in terms of absorbance ratios (AR) (Table 2), not all coating-conjugate combinations having positive values (AR > 2.0) at high bacterial cell concentrations also had AR > 2.0 at all concentrations approaching the limits of sensitivity ($10^5$ cells/ml) of the technique. Only one of nine combinations giving a positive visual estimate at $10^5$ cells/ml also had a positive absorbance ratio. By contrast, four of the seven visually negative combinations had positive ratios. Moreover, at $10^4$ cells/ml none of the three combinations rated visually positive had positive absorbance ratios, while three of the thirteen visually negative combinations did. This apparent discrepancy is related to the fact that each coating-conjugate combination had different control values (Table 1). The effect of this variable background was to greatly reduce apparently large differences in the uncorrected $A_{405}$ values among different combinations (Fig. 3). As a consequence, the absorbance ratios generally increased with increasing conjugate dilutions and decreasing coating concentrations which had lower backgrounds.

**Kinetics of the Reaction**

At all bacterial concentrations the mean $A_{405}$ values increased with an increased incubation time (Fig. 4). With both bacterial strains, the $A_{405}$
Table 1. The effect of coating γ-globulin concentration and enzyme-γ-globulin conjugate dilution on mean $A_{405}$ of controls (buffer only) treatments in an ELISA for **Erwinia carotovora** subsp. **atroseptica**

<table>
<thead>
<tr>
<th>Coating concentration (μg/ml)</th>
<th>Mean $A_{405}$ at conjugate dilution</th>
<th>1:100</th>
<th>1:200</th>
<th>1:400</th>
<th>1:800</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td></td>
<td>0.308</td>
<td>0.221</td>
<td>0.132</td>
<td>0.106</td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td>0.203</td>
<td>0.160</td>
<td>0.114</td>
<td>0.078</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>0.116</td>
<td>0.067</td>
<td>0.023</td>
<td>0.043</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>0.024</td>
<td>0.035</td>
<td>0.014</td>
<td>0.018</td>
</tr>
</tbody>
</table>

*Means (of three replicates) followed by the same letter are not significantly different (P=0.05) according to Tukey's multiple range test.

**Visually negative ($A_{405} \leq 0.140$ not detectable visually)**
Table 2. The effect of coating concentration and enzyme-γ-globulin conjugate-dilution on the absorbance ratios (AR) obtained by ELISA for known concentrations of *Erwinia carotovora* subsp. *atroseptica*

<table>
<thead>
<tr>
<th>Coating concentration (µg/ml)</th>
<th>Conjugate dilution</th>
<th>Conjugate dilution</th>
<th>Conjugate dilution</th>
<th>Conjugate dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:100</td>
<td>1:200</td>
<td>1:400</td>
<td>1:800</td>
</tr>
<tr>
<td>4.0</td>
<td>6.5 8.9 14.6 14.4</td>
<td>3.2 3.8 4.9 4.9</td>
<td>1.3 1.2 1.6 1.5</td>
<td>1.1 1.1 1.3 1.0</td>
</tr>
<tr>
<td>2.0</td>
<td>9.6 12.0 15.2 19.4</td>
<td>4.3 4.5 5.1 6.5</td>
<td>1.2 1.3 1.5 1.4</td>
<td>0.9 1.0 0.7 0.8</td>
</tr>
<tr>
<td>1.0</td>
<td>16.7 28.6 72.7 30.0</td>
<td>6.6 9.9 22.3 10.2</td>
<td>1.4 2.2 4.6 1.8</td>
<td>1.2 1.1 2.4 0.9</td>
</tr>
<tr>
<td>0.5</td>
<td>52.3 29.3 59.1 35.8</td>
<td>14.7 8.7 18.9 11.8</td>
<td>3.1 1.7 3.1 2.0</td>
<td>2.0 0.9 2.1 0.9</td>
</tr>
</tbody>
</table>

*Each AR based on the mean A₄₀₅ of three replicates

**Visually negative (A₄₀₅ < 0.140 were not detectable visually)**
Fig. 3. Effect of coating γ-globulin concentration and enzyme-γ-globulin conjugate dilution (1:100 ■ , 1:200 □□□ , 1:400 □□□□ , 1:800 □□□□□□) on mean absorbance (A405) with 10^5 cells/ml in an ELISA for Erwinia carotovora subsp. atroseptica. Each value is the mean of three replicates. Corrected values = uncorrected-control.
Fig. 4. Effect of reaction time, bacterial strain and cell concentration on absorbance ($A_{0.05}$) in an ELISA for Erwinia carotovora subsp. atroseptica. Each point is the mean $A_{0.05}$ of six replicates.
values were proportional to the bacterial concentrations at $10^6$, $10^7$ and $10^8$ cells/ml. Mean $A_{405}$ values corresponding to $10^3$, $10^6$ and $10^5$ cells/ml were equivalent to the control.

**Washing Procedure Standardization**

Use of a controlled-pressure washing system compared to washing by wash bottle (Table 3) resulted in higher mean $A_{405}$ values at all bacterial concentrations regardless of the washing solution. However, none of the $A_{405}$ values obtained with $10^5$ or fewer cells/ml in either system, with any of the washing solutions, was significantly different from their corresponding controls. Although the differences between washing solutions in the controls were not significantly different, PBS-T gave the highest background $A_{405}$ in both washing systems and the lowest corrected values (Fig. 5). Increases of 28.5%, 28.2% and 19.0% in the corrected $A_{405}$ values were obtained by using the standardized DW rinse for cell concentrations of $10^7$, $10^6$ and $10^5$ respectively. Similarly, increases of 48.5%, 66.6% and 114.5% were obtained with the standardized PBS rinse. By contrast there was either no change ($10^7$ and $10^5$ cells/ml) or a slight decrease ($10^6$ cells/ml) when PBS+T was used.

Drying the plates in a stream of air after each washing had no effect on the mean $A_{405}$ values obtained (Table 4). Regardless of whether the plates were dried or not, a film was observed on the bottom of the wells whenever PBS+T was employed as the washing solution. The mean $A_{405}$ values for the PBS+T controls were significantly higher than the DW or PBS controls. Although the absolute $A_{405}$ values in this experiment were higher than comparable treatments in the previous experiments (Table 3), similar trends were observed. DW and PBS alone gave significantly higher mean $A_{405}$ values than PBS+T at $10^6$ and $10^7$ cells/ml. In this experiment at $10^5$ cells/ml the $A_{405}$
Table 3. The effect of washing system and washing solution on absorbance ($A_{405}$) and absorbance ratio (AR) obtained with known cell concentrations in an ELISA optimized for detection of *Erwinia carotovora* subsp atroseptica

<table>
<thead>
<tr>
<th>Washing system</th>
<th>Washing solution</th>
<th>$10^7$</th>
<th>$10^6$</th>
<th>$10^5$</th>
<th>$10^4$</th>
<th>$10^3$</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$A_{405}$</td>
<td>AR</td>
<td>$A_{405}$</td>
<td>AR</td>
<td>$A_{405}$</td>
<td>AR</td>
</tr>
<tr>
<td>Wash</td>
<td>DW</td>
<td>0.847c**</td>
<td>60.5</td>
<td>0.177 ef</td>
<td>12.6</td>
<td>0.035 ghi</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Bottle PBS</td>
<td>0.835  c</td>
<td>64.2</td>
<td>0.160 f</td>
<td>12.3</td>
<td>0.027 hi</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>PBS+T</td>
<td>0.845  c</td>
<td>14.1</td>
<td>0.209 def</td>
<td>3.5</td>
<td>0.070 ghi</td>
<td>1.2</td>
</tr>
<tr>
<td>Controlled</td>
<td>DW</td>
<td>1.092  b</td>
<td>52.0</td>
<td>0.230.de</td>
<td>11.0</td>
<td>0.046 ghi</td>
<td>2.2</td>
</tr>
<tr>
<td>Pressure</td>
<td>PBS</td>
<td>1.237  a</td>
<td>61.9</td>
<td>0.265 d</td>
<td>13.3</td>
<td>0.050 ghi</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>PBS+T</td>
<td>0.856  c</td>
<td>12.4</td>
<td>0.227 de</td>
<td>3.3</td>
<td>0.091 g</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*Limit of visual estimation

**Means (of six replicates) followed by the same letter are not significantly different (P=0.05) according to Tukey's multiple range test.
Fig. 5. Effect of washing system and washing solution (DW ▼, PBS ▼▼▼, PBS+T ▼▼▼▼) on absorbance (A,05) obtained with known cell concentrations in an ELISA optimized for the detection of Erwinia carotovora subsp. atroseptica. Each value represents the mean of six replicates. Corrected values = uncorrected-control.
Table 4. Effect of washing solution and forced air drying on absorbance ($A_{405}$) and absorbance ratio (AR) obtained with known cell concentrations in an ELISA optimized for detection of *Erwinia carotovora* subsp. *atroseptica*

<table>
<thead>
<tr>
<th>Drying</th>
<th>Washing solution</th>
<th>Absorbance ($A_{405}$)</th>
<th>Absorbance ratio (AR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$10^7$ (AR)</td>
<td>$10^6$ (AR)</td>
</tr>
<tr>
<td>No</td>
<td>DW</td>
<td>1.375 b*</td>
<td>0.463 f</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>1.562 a</td>
<td>0.566 e</td>
</tr>
<tr>
<td></td>
<td>PBS+T</td>
<td>1.091 c</td>
<td>0.392 g</td>
</tr>
<tr>
<td>Yes</td>
<td>DW</td>
<td>1.540 a</td>
<td>0.598 e</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>1.509 a</td>
<td>0.579 e</td>
</tr>
<tr>
<td></td>
<td>PBS+T</td>
<td>0.914 d</td>
<td>0.340 g</td>
</tr>
</tbody>
</table>

*Means (of six replicates) followed by the same letter are not significantly different (P=0.05) according to Tukey's multiple range test.

**Limit of visual estimation.
values for DW and PBS but not PBS+T were significantly greater than their corresponding controls. The limit of detection both visually and by absorbance ratios was $10^5$ cells/ml for DW and PBS but $10^6$ cells/ml for PBS+T.

Three different lots of Tween-20 employed in the PBS+T washing solution, gave higher mean background values ($A_{405}$) than DW and PBS (Table 5). But only Lot-1 at bacterial concentrations of $10^6$ and $10^7$ cells/ml resulted in significantly higher mean $A_{405}$ values than DW or PBS. Absorbance ratios obtained at bacterial concentrations of $10^5$ and $10^7$ cells/ml were greater with DW and PBS than any of the Tween-20 lots employed reflecting the effects of the higher background $A_{405}$ values obtained with Tween-20. The limit of sensitivity was $10^5$ bacterial cells/ml for all washing solutions, both visually and by absorbance ratios.

The duration, pressure and number of rinses at each washing step were all found to influence mean $A_{405}$ values (Table 6). At any pressure with a 5 or 10 sec duration, increasing the number of washes decreased the background values in the control. With combinations involving longer times, the first wash apparently removed everything that could be removed. Similarly at a bacterial concentration of $10^5$ cells/ml and the combinations of 15 sec-68.95 kPa (10 psi)-2 or 3 washes, 20 sec-34.48 kPa (5 psi)-3 washes and 20 sec-68.95 kPa (10 psi)-1,2 or 3 washes, the increased washing resulted in $A_{405}$ values decreased to a level (<0.05) considered too low to be reliably used, compared to the remaining combinations where strong coloration still developed at $10^5$ bacterial cells/ml with very little background in the controls. Two 15 sec washes at 34.48 kPa were selected as the optimum washing conditions because the background was minimized but the sample mean $A_{405}$ values were high at $10^5$, $10^6$ and $10^7$ cells/ml. The limit of visual estimation was $10^6$ bacterial
Table 5. Effect of different washing solutions applied by controlled pressure washing system on the absorbance ($A_{405}$) and absorbance ratios (AR) obtained with known cell concentrations in an ELISA optimized for detection of *Erwinia carotovora* subsp. *atroseptica*

Absorbance ($A_{405}$) and absorbance ratios (AR) for bacterial concentrations (cells/ml)

<table>
<thead>
<tr>
<th>Washing Solution</th>
<th>$10^7$</th>
<th>AR</th>
<th>$10^6$ *</th>
<th></th>
<th>$10^5$</th>
<th>AR</th>
<th>$10^4$</th>
<th>AR</th>
<th>$10^3$</th>
<th>AR</th>
<th>$10^0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW</td>
<td>1.687 b**</td>
<td>29.6</td>
<td>0.464 d</td>
<td>8.1</td>
<td>0.108 ef</td>
<td>1.9</td>
<td>0.059 f</td>
<td>1.0</td>
<td>0.055 f</td>
<td>1.0</td>
<td>0.057 f</td>
</tr>
<tr>
<td>PBS</td>
<td>1.707 b</td>
<td>31.0</td>
<td>0.443 d</td>
<td>8.1</td>
<td>0.098 ef</td>
<td>1.8</td>
<td>0.053 f</td>
<td>1.0</td>
<td>0.058 f</td>
<td>1.1</td>
<td>0.058 f</td>
</tr>
<tr>
<td>PBS+T Lot 1</td>
<td>1.805 a</td>
<td>23.4</td>
<td>0.529 c</td>
<td>6.9</td>
<td>0.128 e</td>
<td>1.7</td>
<td>0.089 ef</td>
<td>1.2</td>
<td>0.080 ef</td>
<td>1.0</td>
<td>0.077 ef</td>
</tr>
<tr>
<td>PBS+T Lot 2</td>
<td>1.651 b</td>
<td>23.9</td>
<td>0.462 d</td>
<td>6.7</td>
<td>0.103 ef</td>
<td>1.5</td>
<td>0.073 ef</td>
<td>1.1</td>
<td>0.067 ef</td>
<td>1.0</td>
<td>0.069 ef</td>
</tr>
<tr>
<td>PBT+T Lot 3</td>
<td>1.708 b</td>
<td>21.1</td>
<td>0.465 d</td>
<td>5.7</td>
<td>0.115 ef</td>
<td>1.4</td>
<td>0.086 ef</td>
<td>1.1</td>
<td>0.078 ef</td>
<td>1.0</td>
<td>0.081 ef</td>
</tr>
</tbody>
</table>

*Limit of visual estimation.

**Means (of six replicates) followed by the same letter are not significantly different (P=0.05) according to Tukey's multiple range test.
Table 6. The effect of number, duration and pressure of distilled water washes applied by a controlled-pressure washing system on the absorbance ($A_{0.05}$) and absorbance ratios (AR) obtained with known cell concentrations in an ELISA optimized for detection of *Erwinia carotovora* subsp. *atroseptica*.

<table>
<thead>
<tr>
<th>Time Pressure sec</th>
<th>Pressure kPa</th>
<th>No. Washes</th>
<th>$10^7$</th>
<th>$10^6$</th>
<th>$10^5$</th>
<th>$10^4$</th>
<th>$10^3$</th>
<th>$10^2$</th>
<th>$10^1$</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$A_{0.05}$</td>
<td>AR</td>
<td>$A_{0.05}$</td>
<td>AR</td>
<td>$A_{0.05}$</td>
<td>AR</td>
<td>$A_{0.05}$</td>
</tr>
<tr>
<td>5</td>
<td>34.48</td>
<td>1</td>
<td>1.131**</td>
<td>37.7</td>
<td>0.427</td>
<td>14.2</td>
<td>0.078</td>
<td>2.6</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.045</td>
<td>41.8</td>
<td>0.367</td>
<td>14.7</td>
<td>0.066</td>
<td>2.6</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.766</td>
<td>40.3</td>
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<td>12.1</td>
<td>0.050</td>
<td>2.6</td>
<td>0.020</td>
</tr>
<tr>
<td>5</td>
<td>68.95</td>
<td>1</td>
<td>0.927</td>
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<td>0.318</td>
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<td>0.078</td>
<td>1.8</td>
<td>0.044</td>
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<tr>
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<td>2</td>
<td>1.117</td>
<td>34.9</td>
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<td>37.0</td>
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<td>11.3</td>
<td>0.052</td>
<td>2.5</td>
<td>0.020</td>
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<tr>
<td>10</td>
<td>34.48</td>
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<td>1.057</td>
<td>22.5</td>
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<td>10</td>
<td>68.95</td>
<td>1</td>
<td>0.821</td>
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<td>0.071</td>
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<td>1.112</td>
<td>41.2</td>
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<td>14.4</td>
<td>0.064</td>
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<td>0.032</td>
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<tr>
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<td></td>
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<td>0.748</td>
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<td>0.219</td>
<td>10.0</td>
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<td>1.662</td>
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<td>0.071</td>
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<td>1.599</td>
<td>319.8</td>
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<td>91.4</td>
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<td>0.006</td>
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<td>1.520</td>
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<td>56.1</td>
<td>0.057</td>
<td>7.1</td>
<td>0.005</td>
</tr>
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<td>68.95</td>
<td>1</td>
<td>1.535</td>
<td>511.7</td>
<td>0.491</td>
<td>163.7</td>
<td>0.054</td>
<td>18.0</td>
<td>0.007</td>
</tr>
<tr>
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<td>1.523</td>
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<tr>
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<td></td>
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<td>328.5</td>
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<td>84.8</td>
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<td>0.001</td>
</tr>
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<td>0.005</td>
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<td>∞</td>
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<td>∞</td>
<td>0.021</td>
<td>∞</td>
<td>0.000</td>
</tr>
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<td>1.573</td>
<td>250.0</td>
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<td>∞</td>
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</tr>
<tr>
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<td>1.257</td>
<td>∞</td>
<td>0.305</td>
<td>∞</td>
<td>0.000</td>
<td>0.0</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
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<td>1.344</td>
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<td>0.357</td>
<td>∞</td>
<td>0.005</td>
<td>∞</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*Limit of visual estimation

**Mean of six replicates
cells/ml for all combinations. Absorbance ratios were greater than 2.0 for all combinations at $10^5$ bacterial cells/ml with the exception of 5 sec-68.95 kPa (10 psi)-1 wash, 10 sec-34.48 kPa (5 psi)-1 wash.

The controlled-pressure DW washing system when applied to a protein antigen system (the Dandelion Virus S assay) also reduced the background compared to the conventional PBS-T wash bottle rinse (Table 7). In this system also, the marked contrast between the uncorrected mean $A_{0.05}$ values and the absorbance ratios for the two washing systems clearly illustrated the importance of thorough washing. This more thorough washing resulted in a 10-fold decrease in the limit of visual estimation in the virus system (Table 11), but a similar increase in sensitivity when absorbance ratios were determined. The bacterial system, included for comparison, had $10^5$ bacterial cells/ml as the visual limit of sensitivity for both washing systems. Based on absorbance ratios the limit of sensitivity was $10^6$ bacterial cells/ml with the conventional system, and $10^5$ bacterial cells/ml with the controlled-pressure DW washing system.
Table 7. Effect of washing method on absorbance ($A_{405}$) and absorbance ratios (AR) obtained with known antigen concentrations in ELISA optimized for the detection of dandelion virus S and *Erwinia* carotovora subsp atroseptica.

<table>
<thead>
<tr>
<th>Washing System</th>
<th>Virus dilution from stock (140 μg/ml)</th>
<th>Bacterial concentration (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^{-1}$</td>
<td>$10^{-2}$</td>
</tr>
<tr>
<td>Wash bottle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$A_{405}$</td>
<td>1.408*</td>
</tr>
<tr>
<td>AR</td>
<td>13.4</td>
<td>4.6</td>
</tr>
<tr>
<td>Standardized, controlled-pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$A_{405}$</td>
<td>0.920</td>
</tr>
<tr>
<td>AR</td>
<td>57.5</td>
<td>19.3</td>
</tr>
</tbody>
</table>

*Mean of six replicates

**Limit of visual estimate
DISCUSSION

The enzyme-linked immunosorbent assay (ELISA) is usually evaluated on the basis of one of two criteria: visual estimates or absorbance ratios >2.0 calculated from spectrophotometric determinations of color development (Lister and Rochow 1979, Voller et al. 1979, Yolken et al. 1977). The former method which is simply qualitative, is gradually being replaced as automated plate reading devices become more available. Both criteria were employed simultaneously in this study which enabled a direct comparison and contrasting of the results obtained by each method. All results were based on determinations made after 30 min which was selected as a reasonable processing time based on previous reports that timing errors could be brought about by shorter times (Bidwell et al. 1977). Prolonging incubation beyond that time to increase the sensitivity as suggested by Engvall and Perlmann (1972), resulted in increased color development in the controls as well as samples which could make visual estimation difficult if not impossible.

Both assessment methods gave similar results when high concentrations of the test antigen were employed. However, at the limits of sensitivity ($10^5$ cells/ml) of the technique different results were obtained. At high coating concentrations and low conjugate dilutions (high conjugate concentrations), visually positive results were recorded at $10^5$ cells/ml. Because the background is a function of coating-conjugate concentrations, the measured backgrounds were higher at these combinations which reduced the possibility of absorbance ratios being > 2.0. At the other extreme, low coating concentrations and dilute conjugates, absorbance ratios > 2.0 were recorded for some samples which were not visually positive (Table 2). Because of the very low
backgrounds obtained at these combinations, any color development or small differences between wells were maximized and probably contributed to the variability observed, and the false positives (AR > 2.0) recorded at cell concentrations considered below the limits of detection.

Because both criteria were employed in the selection of the optimum coating and conjugate dilutions in the Erwinia carotovora subsp. atroseptica system, any combinations which did not give a visible color development in 30 min at the limit of sensitivity (10^5 cells/ml) were discarded. The combinations of 2.0 µg/ml, and 4.0 µg/ml with conjugate dilutions of both 1:100 and 1:200, for which visually positive controls were obtained within 30 min were also discarded (Table 3). The 4.0 µg/ml-1:800 combination was eliminated because of the high background associated with this high coating concentration. Because there was no significant difference between 2.0 and 4.0 µg/ml at 1:400, the lower level was favored for conservation of antiserum as well as high background considerations. Similarly, because there was no significant difference between 1:100 and 1:200 at 1.0 µg/ml, the greater dilution was favored. Thus, the choice of an optimum coating-conjugate combination was narrowed down to 2.0 µg/ml-1:400, and 1.0 µg/ml-1:200. While the latter was the only combination to give both a visual positive, and an AR > 2.0 at a bacterial concentration of 10^5 cells/ml, the 2.0 µg/ml-1:400 combination was selected as a compromise to economize on the conjugated antiserum. As Herrmann and Collins (1976), and Lister and Rochow (1979) have pointed out, conservation of reagents is an important consideration when little is gained by the use of higher concentrations.

The only previous work involving detection of Erwinia carotovora subsp. atroseptica by ELISA (Cother and Vruggink 1980, Vruggink 1978) employed a
combination of 1.0 μg/ml of coating and a conjugate dilution of 1:400, with no mention of the time allowed for the reaction to proceed. Furthermore, because of differences in antiserum preparation (live cells versus glutaraldehyde-fixed cells), and slightly different glutaraldehyde concentrations in the conjugation procedure, which could be critical according to Korpraditskul et al. (1979), the systems cannot be directly compared.

Based on these results, the widespread use of Tween-20 as a component of the washing solution for ELISA is not justified. Its inclusion resulted in higher background $A_{0.05}$ values, and significantly lower sample $A_{4.05}$ values at high bacterial concentrations (10$^6$ and 10$^7$ cells/ml) compared to distilled water or PBS. The consequence was a loss in sensitivity due to the high background which determines absorbance ratios (Table 4, 10-fold loss in sensitivity). This result supports Bruins et al. (1978) who also reported that the use of Tween-20 in the washing solution interfered with the detection of a lipopolysaccharide antigen. However, it does not confirm Bullock and Walls (1977) report that the presence of Tween-20 effectively eliminated background reactions and increased sensitivity in assays for *Toxoplasma gondii* antigen. The film observed on the bottom of all plates washed with Tween-20, could not be related to the quality (age) of the material employed. This film provides an explanation for the occurrence of increased background $A_{4.05}$ values without any increase in visually detected color development. Because the $A_{4.05}$ values were determined directly through the wells, this film could increase background $A_{4.05}$ values without affecting the rate of visibly detectable color in the wells. The reduction in sample $A_{4.05}$ values in spite of the film can also be explained. It is possible that the detergent action of Tween-20 may be removing some of the cells at high bacterial concentrations so that the final
antigen concentration resulted in a reduced $A_{405}$ value which resulted in a net reduction in spite of the film on the plates. Further work will be required to determine whether antigens of different sizes and chemical composition are equally prone to this proposed mechanism. However in this work, both protein and LPS antigens responded similarly to the use of Tween-20 in the washing solution (Table 7).

Because no significant difference was obtained between distilled water and PBS, and because the former proved much easier to use with the controlled pressure washing system, distilled water was adopted as the washing solution. The use of tap water as suggested by Bidwell et al. (1977) and Henrikson (1979) was not considered because of the variability in tap water from one location to another.

The importance of thorough and uniform washing (Birch et al. 1979, Voller et al. 1979) was confirmed in this work. The fact that increases of 19.0% to 38.5% in sample $A_{405}$ values corrected for background were obtained by the standardized method employing distilled water illustrates the importance of a thorough washing. However, as shown by successive washes, sample as well as background can be removed if optimum conditions are not determined. These results support the conclusion of Lehtonen and Viljanen (1980) that the number of washes is critical in determining the final surface concentration on the wells. Because different combinations of pressure and time resulted in different degrees of background and/or antigen removal (Table 6), uniform and reproducible washing should be employed.

A combination involving two washes rather than one longer wash was chosen so that the plate could be rotated 180° on the washer to minimize error. A 15-sec wash at 34.48 kPa (5 psi) in the system employed,
corresponded to about 1600 ml. Henriksen (1979) employed a somewhat similar washing device without pressure control. He reported reproducible results with 10 to 15 sec washings where approximately 1000 ml/5 sec were poured directly on the spreader plate.

When the same treatments were run under apparently similar conditions in successive experiments, the $A_{\text{405}}$ varied considerably. While the trends in the data were consistent, the limit of sensitivity for detection of *Erwinia carotovora* subsp. *atroseptica* varied between $10^5$ and $10^6$ cells/ml, in spite of optimized coating-conjugate conditions, and reproducible washing conditions. Lister and Rochow (1979) have noted similar variability in an ELISA for Barley Yellow Dwarf Virus. Voller et al. (1979) have reported that small departures from precise optimal conditions of pH, time, temperature or reagent concentrations can interfere with the reproducibility of the results. Thus a thorough study of other factors influencing the $A_{\text{405}}$ values in this system should be undertaken to identify additional sources of variability that would interfere with the detection of *Erwinia carotovora* subsp. *atroseptica*. 
LITERATURE CITED


CHAPTER II. FACTORS CONTRIBUTING TO THE VARIABILITY OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ERWINIA CAROTOVORA SUBSP. ATRO-SEPTICA (VAN HALL) DYE.

INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA), is often referred to as an accurate, reliable, and reproducible technique (Bar-Joseph et al. 1979; Birch et al. 1979; Brodeur et al. 1978; Bullock and Walls 1977; Carlsson et al. 1975; Engvall and Perlmann 1971; Gugerli and Gehriger 1980; Kishinevsky and Bar-Joseph 1978; Lister and Rochow 1979; Marco and Cohen 1979; Voller et al. 1976, 1977). These qualities have resulted in the several authors (Clark and Adams; 1977; Engvall et al. 1971; Vruggink 1978) pointing out the quantitative potential of the technique. However, this potential has never been fully realized due to the variation observed between replicates within the same test (Bullock and Walls 1977; Carlsson et al. 1972, 1975; Engvall et al. 1971; Lister and Rochow 1979). Although this variability has been attributed primarily to variations within the microtitration plates (Clark and Adams 1977; Denmark and Chessum 1978; McMurray and Blanchflower 1979), other factors may be involved. Voller et al. (1979) have pointed out that even small departures from the optimal conditions determined for a given system, can interfere with reproducibility of the results.

The limited studies with plant pathogenic bacteria, including Erwinia carotovora subsp. atroseptica (van Hall) Dye (Cother and Vruggink 1980; Vruggink 1978); Corynebacterium sepedonicum (Spieck. & Kotth.) Skapt. & Burkh.
(Claflin and Uyemoto 1978) *Corynebacterium michiganense* (E.F. Sm) Jensen
(Stevens and Tsiantos 1979); *Pseudomonas phaseolicola* (Burkh.) Dawson (Weaver
and Guthrie 1978); *Xanthomonas pelargonii* (N.A. Brown) Starr & Burkh.
(Vruggink 1978) and *Rhizobium* spp. (Berger et al. 1979; Kishinevsky and Bar-
Joseph 1978) have directly applied techniques developed for use with plant
viruses to the bacterial systems. The extreme sensitivity characterizing the
virus assays has not generally been observed with the bacteria. Whether
reaction conditions optimal for plant virus detection are also suitable for
bacterial detection remains to be determined. The large number of different
reaction conditions reported in the more thoroughly-researched systems sug-
gests that comparative studies would be in order even here.

The reaction condition upon which there seems to be the least agreement
in the ELISA literature is the choice of buffers for antigen samples and
enzyme-conjugates. Phosphate buffered saline plus 0.05% Tween-20 (PBS+T) has
been used for sample preparation involving antibodies (Bullock and Walls 1979;
1976) viruses (Crook and Payne 1980) and bacterial enterotoxin (Yolken et al.
1977). PBS+T plus 2.0% polyvinylpyrrolodine (PBS+T+PVP) has been employed for
sample preparation in studies involving plant viruses (Bar-Joseph et al. 1979;
Lister and Rochow 1979; Marco and Cohen 1979; Tresh et al. 1977) and phyto-
pathogenic bacteria (Vruggink 1978). PBS+T+PVP plus 0.2% egg albumin (PBS+T+PVP+EA) has been used with plant viruses (Gugerli and Gehringer 1980) and
phytopathogenic bacteria (Stevens and Tsiantos 1979). Many of these same
buffers have also been employed with the conjugate preparations. Phosphate
buffered saline (PBS) (Birch et al. 1979) and PBS+T have been widely used
(Bullock and Walls 1977; Crook and Payne 1980; Russell et al. 1976; Voller et
al. 1976), PBS+T+PVP (Nachmias et al. 1979) and PBS+T+EA (Vruggink 1978) have also been used.

The objectives of this work were to determine the relative importance of well to well variation in microtitration plates, of different sample and conjugate buffers and of pipetting errors as sources of variability in an ELISA for the detection of Erwinia carotovora subsp. atroseptica.
MATERIALS AND METHODS

Bacterial Cultures, Antiserum Production, γ-globulin Purification, and Alkaline Phosphatase-γ-globulin Conjugation

Potato strains (E82, E193) of _Erwinia carotovora_ subsp. _atroseptica_ conforming to Serogroup I (DeBoer et al. 1979) were employed throughout this study. Stock cultures were maintained on Difco Nutrient Agar (NA) slants at 4°C. Unless otherwise noted, cell suspension from cultures grown for 48 h on NA at 27°C were employed in all tests.

Antiserum against glutaraldehyde-fixed whole cells of strain E82 was prepared in rabbit by the procedure of Allan and Kelman (1977). Crude antiserum was partially purified by saturated ammonium sulfate precipitation and passage through a DEAE-22 Sephadex column prior to conjugation with alkaline phosphatase (Chapter 1).

Basic ELISA Procedure

The basic procedure employed was identical to that previously described (Chapter 1) unless indicated otherwise. The optimum coating γ-globulin concentration of 2.0 μg/ml and conjugation dilution of 1:400 were employed in all experiments. Plates were washed with distilled water for 15 sec at 34.48 kPa (5 psi), rotated 180° on the washing devise and washed a second time. Plates were dried by two 5-sec applications of compressed air (medical grade) at 172.38 kPa (25 psi).

The enzyme substrate reaction was allowed to proceed for 30 minutes at room temperature. A qualitative estimate of color development was made and plates were read spectrophotometrically at 405 nm with a Titertek Multiskan plate reader calibrated at the time of substrate addition. An absorbance
ratio \(\frac{A_{405 \text{ sample}}}{A_{405 \text{ control}}} \geq 2\) was considered a positive result.

**Plate Uniformity**

To determine the well to well variation in the microtitration plates, all 96 wells in each of six plates received the same treatment. A suspension of \(10^5\) cells/ml of *Erwinia carotovora* subsp. *atroseptica* strain E193 was employed. Samples were distributed in the wells using a 8 channel Multi Channel Pipetter. The plates were read spectrophotometrically after 30, 45 and 60 min.

Bacterial dilution series ranging from \(10^3\) to \(10^8\) cells/ml of strains E193 and E82 were prepared. Each dilution of each strain was replicated six times on one microtitration plate, (84 wells) and compared to the sample buffer control. The reaction was assessed immediately after addition of the substrate and every 5 minutes thereafter.

**Effect of Pipetting Errors**

To determine whether pipetting errors due to tip replacement affected ELISA, the standard assay was employed but coating, sample and conjugate were added 10 wells/tip, 5 wells/tip or 1 well/tip. One microtitration plate was employed. Columns 1 and 12 received only the buffers, and the substrate. A bacterial suspension of \(10^6\) cells/ml of strain E193 was employed. Each series of wells was replicated five times.

To determine the effect of pipetting errors at different stages of the assay, an additional 10 \(\mu\)l of coating, sample or conjugate was added indivi-
dually to wells which had already received normal coating, sample or conjugate. One microtitration plate was employed. Columns 1 and 12 received only the buffers, and the substrate. A bacterial suspension of $10^6$ cells/ml (strain E193) was used. Each combination coating, sample and conjugate was replicated 10 times.

**Effect of Different Buffers on ELISA**

To determine the effect of the sample buffer on ELISA, the standard ELISA procedure was followed, except that bacterial suspensions of $10^5$ cells/ml (strain E193) were made up in different buffers. A bacterial suspension adjusted to $A_{540} = 0.1$ ($10^8$ cells/ml) was prepared in sterilized distilled water, and diluted to $10^6$ cells/ml in each of the following: distilled water (DW); phosphate buffered saline (PBS); PBS + 0.05% Tween-20 (polyoxyethylene (20) sorbitan monolaurate, Fisher Scientific) (PBS + T); PBS + 2.0% polyvinylpyrrolidone (M.W. approx. 44000, BDH Chemicals) (PBS+PVP); PBS + 0.2% egg albumin (Egg albumin (ovalbumin) Grade III, Sigma, No-5378) (PBS+EA); PBS+T+PVP; PBS+T+EA; PBS+PVP+EA; and PBS+T+PVP+EA. All treatments were run on one plate. Columns 1 and 12 and Row A from 1 to 5, and Row H from 8 to 12, received only DW and the substrate. Each treatment was replicated eight times. Similarly, to determine the effect of the conjugate buffer on ELISA, the standard procedure was followed, except that conjugate dilutions of 1:400 were prepared in the different buffers described above. The buffers employed, and the plate design were described above. A bacterial suspension of $10^6$ cells/ml (strain E193) in PBS+T+PVP+EA was employed.

A comparison of individual buffers used for both sample and conjugate preparation, was undertaken using a modified standard procedure. In
preliminary experiments only bacterial suspensions of $10^6$ cells/ml (strain E193) and conjugate dilutions of 1:400 were made up in the buffer series listed above. An individual treatment consisted of the same buffer being used for both sample and conjugate preparations. In later experiments a bacterial concentration series ranging from $10^3$ to $10^7$ cells/ml were employed with the appropriate buffer only control. Only 54 wells from each of six microtitration plates were employed. The outside wells, and wells from Row B (1 - 4) and G (9-12) received only DW and the substrate. Each treatment was replicated six times, once per plate.

Statistical Analysis

In each experiment, all treatments or combinations of treatments on one plate, were completely randomized. In some experiments, Tukey's multiple range test was performed on the $A_{405}$ values uncorrected for their controls with a 5.0% significance level for the F-value.
RESULTS

Plate Uniformity

When all wells in six plates were treated identically, the $A_{05}$ values within plates and between plates differed considerably. To determine whether there was any pattern to this variation an acceptable range around the average value equal to ± 10% of the mean was arbitrarily established for each plate (Fig. 1). Some plates showed more uniformity (plates three and six) than others (plates four and five) after a 30 min reaction time. Values falling below the acceptable range were concentrated in the middle part of plates one and two, were located more on the left side of plates three and four, and were generally dispersed on plates five and six. Values above the acceptable range were concentrated in the bottom third of plates four and five, the top third of plates one and six, and more generally distributed on plates two and three. Values both above and below the range were observed in the outside rows, and were more numerous in some plates (plates four and five) than others (plates one and six). Both types of values were frequently found in the same column or row.

The number of wells with values greater or smaller than the 10% limit of acceptable variability decreased as the reaction time increased. The number of wells outside the acceptable range on plate one went from 31 after 30 min, to 20 after 45 min, to 11 after 60 min. Similar decreases in the number of wells were observed with the other plates. As reaction time increased, the distribution of these wells outside the acceptable range changed. Some wells previously within the ±10% range of acceptable variability were now outside, where others previously outside were now within. For
Figure 1. Variability in $A_{05}$ values obtained at three reaction times in an ELISA optimized for detection of Erwinia carotovora subsp. atroseptica. All wells received $10^6$ cells/ml ($\text{•} \geq$ mean plus 10% of mean, $\text{○} <$ mean minus 10% of the mean).
example on plate one (Fig. 1) wells D-5, H-6 and G-11 were within the acceptable range after 30 min but were outside it after 45 min. Conversely wells A-1, E-3, C-4, A-5, C-5, D-6, A-7, E-7, F-8, F-9, F-10, H-11, B-12 and C-12 fell outside the range at 30 min but within it after 45 min. After 60 min all of the above wells plus several others (F-3, C-5, E-5, C-6, E-8, E-9 and H-9) were within the range. If the acceptable range was increased from ± 5% to ± 10% of the mean or to ± 15% of the mean, the number of wells falling outside the range decreased (Fig. 2). The combination of the largest range and the longest incubation period gave a "uniform" plate in one instance but not the other.

In another experiment the maximum percent variation from the mean was determined for A_{05} values read at 5 min intervals. Regardless of strain or bacterial cell concentration, the maximum percent variation decreased with time as the A_{05} mean value increased (Fig. 3). After about 20 min at most bacterial cell concentrations, the maximum percent variation tended to "level-off". An examination of the raw data revealed that a different replicate was usually responsible for the maximum variation at successive readings, suggesting that well to well variability was again the causal factor. The lowest percent maximum variation was associated with treatments giving the highest A_{05} mean values and vice versa. The maximum percent variation observed after 30 min at the limit of detection (10^5 cells/ml) for this experiment was 16.0% for both strains.

Effect of Pipetting Errors

Retention of liquid in the pipette tip was often observed when several wells were filled with the same tip. This retention appeared to add to the
Fig. 2. Well to well variability in $A_{0.05}$ values obtained at different reaction times when all wells received $10^6$ cells/ml in an ELISA optimized for *Erwinia carotovora* subsp. *atroseptica* ($O >$ mean $+$ indicated % of mean; $O <$ mean $-$ indicated % of mean).
Fig. 3. Maximum percent variation from the mean $A_{0.05}$ values obtained at 5-min intervals for known bacterial concentrations of strains E82 and E193 in an ELISA optimized for detection of *Erwinia carotovora* subsp. *atroseptica*.
variability only when 10 wells were filled from the same tip (Table 1). The well to well variation previously noted would account for the variability between wells filled with separate tips and between wells filled in groups of five with the same tip. This interpretation was supported by the fact that in experiments where deliberate pipetting errors of 5% (V/V) were made at all possible points in the assay there were no statistically significant differences obtained between the $A_{405}$ values (Table 2).

**Effect of Different Buffers on ELISA**

Preliminary studies (Table 3) on the effect on $A_{405}$ of different buffers employed for sample preparation or conjugate dilution alone or for both simultaneously, clearly showed that there were differences. Not only was there a buffer requirement for sample and conjugate preparation but there were also significant differences between the buffers employed. Distilled water as a sample diluent resulted in significantly lower mean $A_{405}$ values than all of the other buffers. Inclusion of egg albumin in both PBS and PBS+T as the sample diluent resulted in higher, but not significantly higher, mean $A_{405}$ values. By contrast, inclusion of PVP reduced mean $A_{405}$ values and in one case the reduction was significant. The standard buffer (PBS+T+PVP+EA) included for comparison had the fourth highest mean $A_{405}$ value but was not significantly different from the first three. Distilled water also gave the lowest $A_{405}$ mean value when employed as the conjugate diluent, but was not significantly different from PBS+T, and PBS+T+PVP. By contrast, PBS and PBS+PVP as conjugate diluents gave mean $A_{405}$ values which were significantly higher than all other buffers including the standard PBS+T+PVP+EA. Again distilled water had the lowest mean $A_{405}$ value when it was used to prepare both the sample and the conjugate. However, it was not significantly different
Table 1. Effect of pipetting error on mean $A_{405}$ values obtained with $10^6$ cells/ml in an ELISA optimized for *Erwinia carotovora* subsp. atroseptica

<table>
<thead>
<tr>
<th>Wells filled/tip</th>
<th>Mean $A_{405}$</th>
<th>Highest % variation from mean observed within a set</th>
<th>% variation of the set from mean of all sets</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.382</td>
<td>14.1</td>
<td>8.2</td>
</tr>
<tr>
<td>10</td>
<td>0.376</td>
<td>15.7</td>
<td>6.5</td>
</tr>
<tr>
<td>10</td>
<td>0.367</td>
<td>7.4</td>
<td>4.0</td>
</tr>
<tr>
<td>10</td>
<td>0.307</td>
<td>25.7</td>
<td>13.0</td>
</tr>
<tr>
<td>10</td>
<td>0.332</td>
<td>25.6</td>
<td>5.9</td>
</tr>
<tr>
<td>5</td>
<td>0.343</td>
<td>8.5</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>0.358</td>
<td>10.9</td>
<td>4.7</td>
</tr>
<tr>
<td>5</td>
<td>0.312</td>
<td>6.4</td>
<td>8.8</td>
</tr>
<tr>
<td>5</td>
<td>0.345</td>
<td>7.0</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>0.353</td>
<td>8.5</td>
<td>3.2</td>
</tr>
<tr>
<td>1</td>
<td>0.360</td>
<td></td>
<td>3.7</td>
</tr>
<tr>
<td>1</td>
<td>0.347</td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>0.310</td>
<td></td>
<td>10.7</td>
</tr>
<tr>
<td>1</td>
<td>0.379</td>
<td></td>
<td>9.2</td>
</tr>
<tr>
<td>1</td>
<td>0.340</td>
<td></td>
<td>2.0</td>
</tr>
</tbody>
</table>
Table 2. Effect of 10 µl of additional coating, sample and/or conjugate on the mean $A_{405}$ values obtained with $10^5$ cells/ml in an ELISA optimized for *Erwinia carotovora* subsp. *atroseptica*

<table>
<thead>
<tr>
<th>Coating</th>
<th>Sample</th>
<th>Conjugate</th>
<th>$A_{405}$***</th>
</tr>
</thead>
<tbody>
<tr>
<td>-*</td>
<td>-</td>
<td>-</td>
<td>0.352 a</td>
</tr>
<tr>
<td>+***</td>
<td>-</td>
<td>-</td>
<td>0.361 a</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>0.358 a</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0.346 a</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>0.366 a</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.363 a</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>0.368 a</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.365 a</td>
</tr>
</tbody>
</table>

* - normal treatment
** + received an additional 10 µl
*** Means (of 10 replicates) followed by the same letter are not significantly different (P=0.05) according to Tukey's multiple range test
from several other buffers. The relative differences between the mean $A_{405}$ values when the same buffer was used for both sample and conjugate preparation were very similar to those obtained when the buffers were used only for the conjugate preparation.

When the experiment involving common buffers for sample and conjugate preparation was repeated at several bacterial concentrations with proper controls (Table 4), the use of PBS, and PBS+PVP resulted in extremely high mean $A_{405}$ values for the controls as well as the samples after 30 min. As a consequence, the absorbance ratios (AR) for both treatments were < 2.0 in spite of the extremely high mean $A_{405}$ values, emphasizing the importance of the background in this assay. Similarly, distilled water had a high background relative to the mean $A_{405}$ values obtained at the different bacterial concentrations which prevented its use. At the limits of detection ($10^5$ cells/ml) only buffers containing egg albumin were visually positive after 30 min, and all had AR > 2.0 with the exception of PBS+EA (AR = 1.9). Although the mean $A_{405}$ values differed, the differences were not significant, so that none represented an improvement upon the complete buffer PBS+T+PVP+EA. Both buffer solutions containing Tween-20 but not egg albumin had AR > 2.0 but were not visually positive at $10^5$ cells/ml. Because the criteria for a positive result was an AR > 2.0 and a positive visual reading after 30 min, the limit of detection was considered to be $10^6$ cells/ml for these treatments.
Table 3. Comparison of the effect of different sample and/or conjugate buffers on absorbance ($A_{0.05}$) obtained with $10^6$ cells/ml in an ELISA optimized for detection of *Erwinia carotovora* subsp. *atro septica*

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Sample only</th>
<th>Conjugate only</th>
<th>Both sample &amp; conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_{0.05}$</td>
<td>Rank</td>
<td>$A_{0.05}$</td>
</tr>
<tr>
<td>DW*</td>
<td>0.277 f**</td>
<td>9</td>
<td>0.110 e</td>
</tr>
<tr>
<td>PBS</td>
<td>0.372 bcde</td>
<td>5</td>
<td>0.775 a</td>
</tr>
<tr>
<td>PBS+PVP</td>
<td>0.345 de</td>
<td>7</td>
<td>0.709 a</td>
</tr>
<tr>
<td>PBS+EA</td>
<td>0.399 abc</td>
<td>3</td>
<td>0.291 bc</td>
</tr>
<tr>
<td>PBS+PVP+EA</td>
<td>0.335 e</td>
<td>8</td>
<td>0.244 cd</td>
</tr>
<tr>
<td>PBS+T</td>
<td>0.418 ab</td>
<td>2</td>
<td>0.182 de</td>
</tr>
<tr>
<td>PBS+T+PVP</td>
<td>0.357 cde</td>
<td>6</td>
<td>0.185 de</td>
</tr>
<tr>
<td>PBS+T+EA</td>
<td>0.429 a</td>
<td>1</td>
<td>0.203 d</td>
</tr>
<tr>
<td>PBS+T+PVP+EA***</td>
<td>0.392 abcd</td>
<td>4</td>
<td>0.361 b</td>
</tr>
</tbody>
</table>

*DW = distilled water; PBS = phosphate buffered saline; PVP = polyvinylpyrrolidone; EA = egg albumin; T = Tween-20

**Means (of 8 replicates) sharing the same letter are not significantly different (P=0.05) according to Tukey's multiple range test.

***Buffer employed in standard procedure.
Table 4. Effect of buffers used for sample and conjugate dilution on absorbance (A$_{405}$) and absorbance ratio (AR) obtained with known cell concentrations in an ELISA optimized for Erwinia carotovora subsp. atroseptica

<table>
<thead>
<tr>
<th>Bacterial concentration (cells/ml)</th>
<th>10$^7$</th>
<th>10$^6$</th>
<th>10$^5$</th>
<th>10$^4$</th>
<th>10$^3$</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A$_{405}$</td>
<td>AR</td>
<td>A$_{405}$</td>
<td>AR</td>
<td>A$_{405}$</td>
<td>AR</td>
</tr>
<tr>
<td>DW*</td>
<td>0.212</td>
<td>k**</td>
<td>1.3</td>
<td>0.138</td>
<td>l</td>
<td>0.9</td>
</tr>
<tr>
<td>PBS</td>
<td>1.604</td>
<td>bc</td>
<td>1.5</td>
<td>1.269</td>
<td>efg h</td>
<td>1.2</td>
</tr>
<tr>
<td>PBS+PVP</td>
<td>1.951</td>
<td>1.9</td>
<td>1.475</td>
<td>cdef</td>
<td>1.5</td>
<td>1.250</td>
</tr>
<tr>
<td>PBS+EA</td>
<td>1.435</td>
<td>cdef</td>
<td>21.4</td>
<td>0.506</td>
<td>j</td>
<td>7.6</td>
</tr>
<tr>
<td>PBS+PVP+EA</td>
<td>1.524</td>
<td>bcde</td>
<td>40.1</td>
<td>0.506</td>
<td>j</td>
<td>14.7</td>
</tr>
<tr>
<td>PBS+T</td>
<td>1.468</td>
<td>cdef</td>
<td>50.6</td>
<td>0.414</td>
<td>jk</td>
<td>14.3</td>
</tr>
<tr>
<td>PBS+T+PVP</td>
<td>1.483</td>
<td>cdef</td>
<td>44.9</td>
<td>0.421</td>
<td>jk</td>
<td>12.8</td>
</tr>
<tr>
<td>PBS+T+EA</td>
<td>1.763</td>
<td>ab</td>
<td>55.1</td>
<td>0.637</td>
<td>j</td>
<td>19.9</td>
</tr>
<tr>
<td>PBS+T+PVP+EA</td>
<td>1.576</td>
<td>bcd</td>
<td>38.4</td>
<td>0.482</td>
<td>j</td>
<td>11.8</td>
</tr>
</tbody>
</table>

*DW = distilled water; PBS = phosphate buffered saline; PVP = polyvinylpyrrolidone; EA = egg albumin; T = Tween-20.

**Means (of six replicates) sharing the same letter are not significantly different (P=0.05) according to Tukey's multiple range test.

***Limit of visual detection.
DISCUSSION

The well to well variability associated with the microtitration plates was found to be the major source of variation in the detection of *Erwinia carotovora* subsp. *atroseptica* by ELISA, confirming previous observations of Clark and Adams (1977), Denmark and Chessum (1978), and McMurray and Blanchflower (1979). The degree of variability was shown to be a function of each individual plate (Figure 1). Outside rows did not give mainly higher values as reported by Denmark and Chessum (1978). Moreover, values outside the arbitrarily set acceptable limits of variability were found not only in the outside rows, but anywhere on the plates. This variability was also a function of reaction time because the pattern was constantly changing on a plate. In addition, these changes occurred in both directions. When increasingly larger acceptable levels of variability were applied, a decrease in the number of wells outside the acceptable limits was obtained for each plate. Increasing the reaction time at any one level of variability also reduced the number of wells outside the limits. At the normal reaction time of 30 min, even with the ±15% limit of variability from the mean, some wells still remained outside. The use of even longer reaction times did reduce the number of wells outside the ±15% limits, but the controls became positive visually after 30 min, which is important in laboratories where only a visual estimate is possible.

The maximum percent variation from the mean varied with time and to a lesser extent with the concentration of bacterial cells employed. Greater percent variation was associated with low bacterial cell concentrations and vice versa. Such a result is consistent with the fact that a small mean value is used for comparison in the first instance and large one in the second. The
decrease with time was expected because the means were increasing as the reaction proceeded and color development intensified. What is not readily explainable is why the maximum percent variation appeared to decrease less rapidly or level-off after about 20 min. The source of the variation is not representable by a constant $A_{405}$ value which decreases in relation to an increasing mean. Instead it appears to be a certain proportion of the ever changing $A_{405}$ values associated with each different bacterial cell concentration. If increased maximum variability is somehow associated with an antiserum excess which is inevitable at the limits of sensitivity, the cause of the variability must be identified and eliminated by means that remain to be determined if the potential of the ELISA is to be fully realized.

At a bacterial concentration of $10^6$ cells/ml, the maximum percentage variability observed for both bacterial strains after a 30-min reaction time was 16%. Other workers have reported variability in the order of 17% for four readings (Bullock and Walls 1977), 10% for duplicates (Carlsson et al. 1972), less than 10% using duplicate samples over a limited antigen range (Clark 1981), 10 to 20% (Carlsson et al.: 1975), and plus or minus 9% from the mean of triplicates after correction was made for the background (Engvall et al. 1971). Thus the variability reported in these experiments is typical and not peculiar to the Erwinia carotovora subsp. atroseptica system.

While conceding that the outside rows were indeed variable, avoiding their use in an experiment design seems unwarranted, because similar variability is also common in other sections of the plate. Replication can reduce the effects of variability. If more than one plate is necessary for a test, it is essential that each plate have its own control, and that the number of replicates be evenly distributed between the plates to minimize both within and
between plate variability. If the appropriate design precautions are taken, the number of plates to be used, should not be considered a limitation where precision is needed.

Investigation of pipetting errors as a probable source of variability showed that it is rather difficult to dissociate the variability resulting from the repeated use of one tip, from the well to well variability of the microtitration plate. The probability of an "odd" well being included in a 10-well series is greater than that in a 5-well series. Furthermore, the variability obtained when only one well is filled per tip could be attributed to either a well or tip effect. The failure to reproduce the observed variability by deliberate pipetting errors equal to 5%, supports the interpretation that well variation not pipetting errors are responsible.

The benefits of sample preparation in a suitable buffer are obvious because of the differences which were demonstrated in this work. High A_{405} values were obtained in the *Erwinia carotovora* subsp. *atroseptica* ELISA only when PBS + 0.05% Tween-20 was employed for sample preparation, presumably because the Tween-20 prevented nonspecific adsorption (Bullock and Walls: 1977, Clark and Adams: 1977, Engvall and Perlmann: 1972, Yolken et al.: 1977). Inclusion of egg albumin which also prevents nonspecific retention of antibodies (Gugerli and Gehriger: 1980), further increased this effect although not significantly. The complete buffer frequently used (PBS+T+PVP+EA) gave a lower mean A_{405} value which would appear to be due to the PVP component. Clark and Adams (1977) also observed that PVP decreased sensitivity. PVP probably could be omitted in further studies involving cultured cells because its role is to remove phenolic compounds present in plant sap which may affect antigen and antibody stability.
The need for buffering capacity and reagents preventing nonspecific absorption in the diluent for pure cultures was shown by the lower mean \( A_{405} \) values obtained when cell suspensions were made up in distilled water. Whether similar reductions in \( A_{405} \) values would be observed if samples were prepared from infected plants remains to be determined. Cother and Vruggink (1980) and Vruggink (1978) have reported detecting \( E. \) carotovora subsp. atroseptica in potatoes by adding a peel extract directly to the wells. Unfortunately no comparisons were made with buffered suspensions of pure cultures. Thus, the effect of plant sap on the detection of this bacterium by ELISA remains to be determined. If the same effect occurs in this system as has been observed in plant virus assays (Clark and Adams 1977; Flegg and Clark 1979), a reduction in sensitivity can be expected.

When different buffers were used only in the preparation of the conjugate dilutions or for the preparation of both the samples and conjugates, the same relative differences between the different buffers were obtained. This suggests that the buffer has a greater effect on ELISA when it is the diluent for the conjugate rather than for the sample. This also suggests that the requirements for binding of bacterial cells to coating \( \gamma \)-globulin may be different from these for the binding of enzyme-\( \gamma \)-globulin to the bacterial cells. The use of buffers not containing either Tween-20 or egg albumin resulted in high backgrounds which were presumably due to nonspecific adsorption. The inclusion of egg albumin in the sample and conjugate buffer is important in the \( E. \) carotovora subsp. atroseptica ELISA, as only those buffers containing it were visually positive after 30 min and had absorbance ratios \( > 2.0 \) (with one borderline exception). Based on these results with bacterial cells grown in culture, there seems to be no compelling reason to
adopt a different buffer system than that currently used in plant virus ELISA systems. A similar study should be done with bacterial cells in plant sap, to determine whether the complete buffer (PBS+T+PVP+EA) is also optimal when plant tissue extracts are involved. However, the possibility exists that the high well to well variation observed in this study masked buffer effects. If the new, improved microtitration plates which became available after this work was completed are really less variable as reported (Clark 1981), a reevaluation of the effect of buffers and pipetting errors would be in order.
LITERATURE CITED


CHAPTER III. SPECIFICITY AND SENSITIVITY OF THE ERWINIA CAROTOVORA SUBSP. ATROSEPTICA (VAN HALL) DYE (SEROGROUP I) ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) has been employed extensively in clinical pathology to detect the presence of a wide range of organisms including protozoa, bacteria and viruses (Voller et al. 1977). In plant pathology, the technique was adapted for virus detection (Clark and Adams 1977), and has been widely employed for a variety of viruses (Bar-Joseph et al. 1979; Flegg and Clark 1979; Gugerli 1979; Gugerli and Gehriger 1980; Lister and Rochow 1979; Marco and Cohen 1979; Tamada and Harrison 1980; Tresh et al. 1977).

The detection of bacterial cells by ELISA has only relatively recently been tried. The medical literature contains numerous examples where bacterial pathogens are being detected by ELISA, but detection is based on detecting the antibodies in human sera rather than the bacteria per se (Brodeur et al. 1978; Russel et al. 1976; Carlsson et al. 1975, 1976; Holmgren and Svennerholm 1973; Ito et al. 1980). Thus, the adaptation of the technique for detection of bacterial cells has involved plant-pathogenic bacteria.

Berger et al. (1979) employed an indirect ELISA to detect whole Rhizobium cells from cultures and pooled nodule samples. They reported that cells produced in broth culture which were washed to remove most of the extracellular polysaccharide slime, reacted qualitatively in the same manner as unwashed cells from plant nodules where no slime was produced. They suggested that the
the extracellular slime when present might interfere with the reaction. They also reported that weak cross reactions were obtained with heterologous antisera. Both Berger et al. (1979) and Kishinevsky and Bar-Joseph (1978), reported that heat treatment of the *Rhizobium* cells improved the reaction in ELISA. Furthermore, Kishinevsky and Bar-Joseph (1978) pointed out that the $A_{405}$ values for the unheated cell suspensions were lower so that strains were readily detected only at high cells concentrations ($10^6 - 10^8$ cells/ml), compared to $10^4 - 10^5$ cells/ml for heated cell suspensions. They also reported that the serological specificity of the *Rhizobium* strains obtained with ELISA generally followed that observed in the agglutination and immunodiffusion tests.

In the preliminary work with plant pathogenic bacteria, the technique has not shown much promise to this point, especially with regard to the level of sensitivity. Weaver and Guthrie (1978) considered $10^6 - 10^7$ cells/ml a high level of sensitivity for *Pseudomonas phaseolicola* (Burkh.) Dawson, and reported cross reactions with other unspecified species. Similarly Vruggink (1978) reported cross reactions between *Xanthomonas pelargonii* (N.A. Brown) Starr & Burkh. (*X. campestris* pv. *pelargonii*) and *Aplanobacter populi* with ELISA. Stevens and Tsiantos (1979) used ELISA to detect whole cells of *Corynebacterium michiganense* (E.F. Sm.) Jensen both in suspensions of known concentration and in tomato plant extracts. They reported that bacteria from culture could be detected at $10^5$ cells/ml. However, their data do not support this conclusion if absorbance ratios $> 2.0$ are used as the criterion for a positive test. A level of $10^5 - 10^6$ cells/ml seems more appropriate from their data. Claflin and Uyemoto (1978) detected whole cells of *Corynebacterium sepedonicum* (Spieck. & Kotth.) Skapt. & Burkh. from infected stems and
tubers of potato and in suspension of cells grown on artificial media. Unfortunately no level of sensitivity was reported. No cross reactions were reported with species of Corynebacterium, Erwinia, Xanthomonas, Pseudomonas or Agrobacterium. The absence of a follow-up paper substantiating this preliminary work casts some doubt on the reliability of this report.

The only instance where ELISA has been used with some success is with Erwinia carotovora subsp. atroseptica. Vruggink (1978), using the general procedure employed for plant virus detection, was able to detect this bacterium in plant material with a γ-globulin coating concentration of 1 μg/ml and a conjugate dilution of 1:400. He cautioned against the use of the technique for a new pathogen without determining specificity and reported that some E. carotovora subsp. carotovora strains reacted with E. carotovora subsp. atroseptica in ELISA. This caution is even more relevant because DeBoer (1980) has recently shown that serological relationships exist among flagellar and somatic antigens of several Erwinia carotovora serogroups.

Cother and Vruggink (1980) have shown that both live and dead cells of Erwinia carotovora subsp. atroseptica reacted in ELISA. They also noted that the lower limit of detection of Erwinia carotovora subsp. atroseptica by ELISA was of 10^4 cells/ml, but did not include any data supporting their conclusion.

The ELISA technique as used to date for detecting whole bacterial cells has not generally shown the high degree of sensitivity and specificity associated with its use for plant viruses. Because of this fact, the objectives of this work were to determine the effect of culture medium, washing bacterial cells and heat treatment on the sensitivity and specificity of detection of Erwinia carotovora subsp. atroseptica by ELISA. A further objective was to determine whether the serogroups, distinguished by immunodiffusion, were detectable by ELISA.
MATERIALS AND METHODS

Bacterial Cultures, Antiserum Production, \( \gamma \)-globulin Purification, and Alkaline Phosphatase-\( \gamma \)-globulin Conjugation

Previously isolated potato strains of \textit{Erwinia carotovora} subsp. \textit{carotovora} (Ecc) and \textit{Erwinia carotovora} subsp. \textit{atroseptica} (Eca) were employed throughout this study. Unless indicated otherwise, bacterial suspensions were prepared from 48-h cultures grown at 27°C on Nutrient Agar.

Antiserum against glutaraldehyde-fixed whole cells of strain E82 was prepared in rabbit by the procedure of Allan and Kelman (1977). Crude antiserum was partially purified by saturated ammonium sulfate precipitation and passage through a DEAE-22 Sephadex column, prior to conjugation with alkaline phosphatase (Chapter 1).

Basic ELISA Procedure

The basic procedure employed was identical to that previously described (Chapter 1) unless indicated otherwise. The optimum coating \( \gamma \)-globulin concentration of 2.0 \( \mu \)g/ml and conjugation dilution of 1:400 were employed in all experiments. Plates were washed with distilled water for 15 sec at 34.48 kPa (5 psi), rotated 180° on the washing device and washed a second time. Plates were dried by two 5 second applications of medical air at 172.38 kPa (25 psi).

The enzyme substrate reaction was allowed to proceed for 30 minutes at room temperature. A qualitative estimate of color development was made and plates were read spectrophotometrically at 405 nm with a Titertek Multiskan plate reader calibrated at the time of substrate addition. An absorbance ratio \( \frac{A_{05 \text{ sample}}}{A_{05 \text{ control}}} \geq 2.0 \) was considered a positive result.
Effects of Culture Conditions on ELISA

To determine the effect of medium, and age of culture on ELISA, strain E82 and E193 of *Erwinia carotovora* subsp. *atroseptica* (*Eca*) conforming to serogroup I (DeBoer et al., 1979), were grown for 48 h at 27 C on five different media: Difco Nutrient Agar (NA), Nutrient Sucrose Agar (NSA), (NA + 5% sucrose), Casamino Peptone Glucose agar (CPG) (Allan and Kelman 1977), King's Medium B (KMB) (King et al. 1954) and Difco Potato Dextrose Agar (PDA). To ensure uniform growth, cells of 48-h cultures on NA were used to inoculate the plates. Ten-fold dilution series in phosphate buffered saline plus 0.05% Tween-20 (polyoxyethylene (20) sorbitan monolaurate, Fisher Scientific) plus 2.0% polyvinylpyrrolidone (M.W. approx. 44000, BDH Chemicals) plus 0.2% egg albumin (Egg albumin (ovalbumin) Grade III, Sigma, No-5378) (PBS+T+PVP+EA) were prepared from $10^4$ to $10^7$ cells/ml, with a control for each dilution series (strain). Each treatment was replicated six times, one replicate/plate. On each plate, the outside rows, and row B from 2 to 6, and row G from 7 to 11 received only the buffers and substrate.

Effect of Heat Treatment of Bacterial Cells on ELISA

To determine the effect of heat treatment of the bacterial cells on ELISA, strain E331 (Serogroup I) of *Eca* was employed. A ten-fold dilution series ($10^3$ to $10^7$ cells/ml) was prepared and a sample buffer only control was included. Each cell concentration was further divided into 1.0 ml aliquots. Four aliquots of each cell concentration were placed in each of five water baths adjusted to 21 C (room T), 40 C, 60 C, 80 C, and 100 C. One tube of each cell concentration at each temperature was removed after 3, 6, 9, and 12 min heating. The samples were allowed to cool at room temperature. Each treatment combination of temperature and time was replicated six times, one
replicate/block, where a block consisted of two plates. Twelve plates were employed, in which the outside rows received the buffers and substrate only. In a subsequent experiment, temperatures of 21°C (room T), 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C were applied for 3 and 6 min. A dilution series ($10^3$ to $10^6$ cells/ml) of strain E193 was similarly prepared, and divided into 2.0 ml aliquots. A buffer only control was similarly treated. Each treatment combination of temperature, and time was replicated six times, one replicate/plate. On each plate, columns 1 and 12, and row A from 2 to 6, and row H from 7 to 11 received only the buffers and substrate.

**Effect of Washing Bacterial Cells on the ELISA**

To determine the effect of washing bacterial cells on ELISA, cells of strain E95 (Serogroup III) of *Ecc* and strain E193 (Serogroup I) of *Eca*, grown for 48 h at 27°C on NA were suspended in PBS+T+PVP+EA. A sample of each was adjusted to $A_{540} = 0.1$ ($10^8$ cells/ml), to serve as the normal, unwashed suspension (normal). The remaining suspensions ($= 10^{10}$ cells/ml) were mixed on a Vortex mixer for 30 sec, and centrifuged at $2.5 \times 10^4$ g for 10 min (Rotor No. 870; IEC Refrigerated Centrifuge Model B-20). The supernatants (cell wash fluids 1) were filtered sterilized (0.22μ) (Millipore Corporation, Bedford, Massachusetts). The pellets were resuspended in 3.0 ml of the sample buffer and a portion of each adjusted to $A_{540} = 0.1$ to give a once-washed cell suspension. This washing step was similarly repeated on the remainder of each suspension to give cell wash fluids 2. A portion of each twice-washed, pellet was adjusted to $A_{540} = 0.1$ to give twice-washed cell suspensions. A dilution series representing concentrations of $10^3$ to $10^8$ cells/ml was tested for each cell suspension. A ten-fold dilution series of each supernatant (undiluted to $10^{-5}$) was similarly tested. Sample buffer only controls were included for
each dilution series. Each treatment was replicated six times, once per plate. Columns 1 and 12 and row A from 2 to 6 and row H from 7 to 11 received only the buffers and substrate.

To determine whether heat labile factors were responsible for the observed reactions, the same bacterial cell washing procedure was repeated with strain E193. Serial dilutions of the supernatants (0 to $10^{-4}$) and of the resuspended washed cells ($10^3$ to $10^7$ cells/ml) were divided into two sets. One set was heated in a water bath at 80°C for 15 minutes then allowed to cool back to room temperature. The control set was similarly treated but at 21°C. Each dilution of the heat-treated supernatants and the washed cells were individually tested in the ELISA. Each treatment was replicated six times, one replicate/plate. The outside rows of each plate received the buffers and substrate only. In a subsequent experiment, similarly prepared supernatants and washed cell suspensions ($A_{540} = 0.1$) of strain E193 were employed undiluted. A sample buffer control was included for each supernatant and cell suspension. A portion of each supernatant or cell suspension (3.0 ml) was autoclaved at 121°C for 90 minutes. Untreated samples were maintained at room temperature. Each treatment was tested individually with five replicates/plate. Columns 1 and 12 received only the buffers and substrate.

**Specificity of ELISA to Different Strains of Erwinia carotovora**

Representative bacterial strains of all 18 *Erwinia carotovora* serogroups determined by DeBoer *et al.* (1979), and 6 new serogroups (S.H. DeBoer personal communication) were employed. One strain of each of *Pseudomonas marginalis* (Brown) Stevens (PM6 - kindly supplied by Dr. A. Kelman, University of Wisconsin) and *Corynebacterium sepedonicum* were added. All strains were grown for 48 hours at 27°C on NA, with the exception of *C. sepedonicum* which was grown
on NM medium (Katznelson and Sutton 1956) for 4 days at 27 C. Bacterial concentrations of $10^8$ cells/ml were employed, to maximize the detection of a reaction. All wells except those in column 1 which received only the buffers and substrate, on two plates were employed. Each strain, and control buffer sample were replicated three times on each plate.

To determine the effect of heat treatment on specificity, varying concentration of only those strains found to react in the previous experiment were heat treated. A non reacting strain (S62) was included as a control. The strains of Eca employed were: E193 (Serogroup I), E19 (Serogroup XVIII), E368 (Serogroup XX), and E555 (Serogroup XXII); while the Ecc strains were: S21 (Serogroup II), E95 (Serogroup III), E14 (Serogroup IV), S26 (Serogroup V), and S62 (Serogroup VIII). A ten-fold dilution series ($10^4$ to $10^7$ cells/ml) and a buffer only control were prepared for each strain. Aliquots (2.0 ml) of each dilution series were placed in water baths at 21 C (room T) and at 60 C for 5 min. After cooling at room temperature, each dilution-temperature combination was considered a treatment for testing by ELISA. Each treatment was replicated six times, 1 replicate per plate. On each plate, columns 1 and 12 received only the buffers and substrate.

**Statistical Analysis**

In each experiment, all treatments or combination of treatments on one plate, were always completely randomized. In some experiments Tukey's multiple range test was performed on the $A_{405}$ values uncorrected for their controls, with a 5.0% significance level for the F-value.
RESULTS

The culture medium upon which the bacteria were grown affected the $A_{405}$ mean values obtained when known concentrations of Eca were tested by ELISA (Table 1). With strain E193, cells grown on NSA had a significantly higher $A_{405}$ mean value at both $10^6$ and $10^7$ cells/ml than cells produced on all other media. By contrast, $10^7$ cells/ml of strain E82 grown on NA gave the highest $A_{405}$ mean value which was significantly different only from that of PDA, and KMB. However, at $10^6$ cells/ml the highest mean $A_{405}$ value was obtained with cells grown on NSA instead of NA, although the difference between them was not significant. Only mean $A_{405}$ values obtained with cells grown on NSA, and CPG were still significantly different from those on KMB. At lower bacterial concentrations, with both strains, there were no significant differences between the mean $A_{405}$ values obtained with cells grown on any of the media, although cells grown on NSA consistently had the highest $A_{405}$ value with both strains. Positive visual estimates and $AR > 2.0$ were obtained with $10^5$ cells/ml of both strains grown in all of the media. At $10^6$ cells/ml, the visual estimates obtained with strain E193 were more variable, and only cells grown on NSA resulted in an $AR > 2.0$. With the homologous strain E82, $10^6$ cells/ml grown on NA, NSA, and CPG resulted in both a positive visual estimate and $AR > 2.0$. The mean $A_{405}$ values for strain E82 compared to those of strain E193 at $10^7$ cells/ml, were significantly greater when the cells were grown on NA, CPG, and PDA but not on NSA or KMB. At $10^6$ cells/ml, only cells grown on CPG gave significantly higher mean values with strain E82. At $10^6$ and $10^5$ cells/ml the mean $A_{405}$ values for both strains grown on all media were not statistically different.
Table 1. Effect of culture media on absorbance ($A_{0.05}$) and absorbance ratio (AR) obtained with known concentrations of 48-h cells of strains E82 and E193 in an ELISA optimized for the detection of Erwinia carotovora subsp. atroseptica

<table>
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<th>$10^6$</th>
<th></th>
<th>$10^5$</th>
<th></th>
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<td>AR</td>
<td>$A_{0.05}$</td>
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<td>$A_{0.05}$</td>
<td>AR</td>
<td>$A_{0.05}$</td>
<td>AR</td>
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<td>0.391 ijkl</td>
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<td>0.142 mno</td>
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<td>0.717 ef</td>
<td>15.6</td>
<td>0.234 klmno</td>
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<td>0.105 o</td>
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*Means (of six replicates) sharing the same letter are not significantly different (P=0.05) according to Tukey's multiple range test.

**Absorbance ratios based on control (mean of six replicates) of $A_{0.05} = 0.046$.

***Limit of visual detection.
Table 2. Effect of heat treatment on the absorbance ($A_{0.05}$) and the absorbance ratios (AR) obtained with known cell concentrations in an ELISA optimized for *Erwinia carotovora* subsp. *atroseptica*

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<td>AR</td>
<td>$A_{0.05}$</td>
<td>AR</td>
<td>$A_{0.05}$</td>
<td>AR</td>
<td>$A_{0.05}$</td>
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<tr>
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<td>3</td>
<td>1.642</td>
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<td>1.120</td>
<td>31.1</td>
<td>0.272</td>
<td>7.6</td>
<td>0.054</td>
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<td>0.036</td>
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<td>6</td>
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<td>66.4</td>
<td>1.201</td>
<td>38.7</td>
<td>0.257</td>
<td>8.3</td>
<td>0.051</td>
<td>1.6</td>
<td>0.037</td>
<td>1.2</td>
<td>0.031</td>
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<td>9</td>
<td>2.089</td>
<td>58.0</td>
<td>1.161</td>
<td>32.3</td>
<td>0.264</td>
<td>7.3</td>
<td>0.057</td>
<td>1.6</td>
<td>0.039</td>
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<td>0.036</td>
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<td>63.6</td>
<td>1.269</td>
<td>39.7</td>
<td>0.227</td>
<td>7.1</td>
<td>0.061</td>
<td>1.9</td>
<td>0.033</td>
<td>1.0</td>
<td>0.032</td>
<td></td>
</tr>
</tbody>
</table>

*Limit of visual detection.

**Mean of six replicates
The amount of growth of 48 h cultures also varied with the medium, and the strain. With both strains the growth on KMB > NSA > NA > CPG > PDA. On KMB both strains produced large mucoid colonies while on NSA lesser amounts of these materials were produced. On NA, CPG and PDA strain E82 gave small, well-isolated, individual colonies. Strain 193 produced similar colonies on CPG and PDA, but gave slightly larger colonies on NA, with very little mucoid materials. Thus, it appears that the type of growth may be important in this assay.

Bacterial suspensions heated to a temperature of 60 C or greater prior to testing by ELISA, had greatly increased mean $A_{405}$ values compared to similar unheated suspensions (Table 2). However, heating did not alter the buffer controls. All combinations of temperature and time gave visually detectable reactions at $10^5$ cells/ml and had AR > 2.0 (Table 2). The absorbance ratios of heat-treated cells were approximately three times those of the corresponding untreated samples at the limit of detection. Subsequent experiments with an expanded temperature range around the critical temperature (Fig. 1) confirmed the initial result that 60 C was the critical minimum temperature required for an increase in $A_{405}$ values. Prolonging the heat treatment at the critical temperature beyond the minimum period tested (3 min) did not further enhance the increase in mean $A_{405}$ observed after the minimum treatment (Table 2 and Fig. 1 and 2).

Washing the cells of strain E193 with distilled water prior to testing resulted in a decrease in the mean $A_{405}$ values and absorbance ratios of the once-washed cells compared to the unwashed suspension (Table 3). The high absorbance ratios observed with even 100-fold dilutions of the wash fluid suggested that at least one of the antigens being detected was soluble. A
Fig. 1. Effect of heat treatment (temperature and duration) on absorbance ($A_{405}$) obtained in an ELISA with known concentrations of *Erwinia carotovora* subsp. *atroseptica*.
Fig. 2. Effect of heat treatment (60°C) duration on absorbance ($A_{405}$) in an ELISA with known concentrations of *Erwinia carotovora* subsp. atroseptica.
second washing did not result in a further decrease in absorbance ratio for
the twice-washed bacterial cells but the undiluted second wash fluid had an
absorbance ratio similar to that of the first wash fluid. Presumably the
concentration of the soluble antigen in this second wash fluid was less
because the ability to detect it was lost at a lower dilution. By contrast
washing cells of strain 95 (Ecc) did not result in increased absorbance ratios
being associated with the wash fluids. One washing did reduce the absorbance
ratios observed for the washed cells but a second washing caused no further
reduction. With both strains there was good agreement between treatments
judged positive on the basis of absorbance ratios greater than 2 compared with
visual estimates.

Heating the wash fluids and cell suspensions for 15 min at 80°C prior to
testing resulted in decreased absorbance ratios for the cell wash fluids but
increased ratios for the washed cell suspensions (Table 4). The limits of
detection for the original suspension increased from $10^6$ to $10^9$ cells/ml on
the basis of absorbance ratios and from $10^7$ to $10^6$ cells/ml visually. A ten-
fold increase in sensitivity was observed both visually and spectrophotometri-
cally when the once-washed cell suspensions were similarly heated. Even
greater increases were observed with the twice-washed cell suspensions. The
absorbance ratios of the wash fluids were decreased by heating but the limit
of detection by absorbance ratios remained unchanged with the exception of the
second cell wash fluid at a dilution of $10^{-4}$ (AR < 2.0). By contrast heat-
ing caused a 10 to 100-fold decrease in the visually determined limit of
sensitivity. The presence of heat-labile antigens in both the wash fluids and
the washed bacterial suspensions was indicated by the decreased mean $A_{0.05}$
values for all samples receiving the 90 min autoclave treatment (Table 5).
Table 3. Detection by ELISA of Erwinia carotovora subsp. atroseptica antigens in distilled water cell wash fluids and washed cell suspensions of subsp. atroseptica (strain E193) and subsp. carotovora (strain E95)

<table>
<thead>
<tr>
<th>Strain Treatment</th>
<th>Absorbance ratio (AR) at dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>undiluted AR</td>
</tr>
<tr>
<td>E193 normal cell* suspension</td>
<td>38.5**</td>
</tr>
<tr>
<td>E193 first wash fluid</td>
<td>39.7</td>
</tr>
<tr>
<td>E193 once-washed cell* suspension</td>
<td>23.9</td>
</tr>
<tr>
<td>E193 second wash fluid</td>
<td>37.7</td>
</tr>
<tr>
<td>E193 twice-washed* cell suspension</td>
<td>21.3</td>
</tr>
<tr>
<td>E95 normal cell* suspension</td>
<td>5.7</td>
</tr>
<tr>
<td>E95 first wash fluid</td>
<td>1.5</td>
</tr>
<tr>
<td>E95 once-washed cell* suspension</td>
<td>2.1</td>
</tr>
<tr>
<td>E95 second wash fluid</td>
<td>1.6</td>
</tr>
<tr>
<td>E95 twice-washed* cell suspension</td>
<td>2.3</td>
</tr>
</tbody>
</table>

*Equivalent bacterial concentrations in cells/ml: undiluted = 10^8, 10^-1 = 10^7; 10^-2 = 10^6; 10^-3 = 10^5; 10^-4 = 10^4; 10^-5 = 10^3.

**Based on average control value of six replicates A_405 = 0.040.

***Limit of visual detection.
Table 4. Effect of a 15 min 80 C heat treatment on detection by ELISA of Erwinia carotovora subsp. atroseptica antigens in distilled water cell wash fluids and washed suspensions of (strain E193)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temp C</th>
<th>Absorbance ratio (AR) at dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>undiluted</td>
</tr>
<tr>
<td>Normal cell suspension**</td>
<td>21</td>
<td>7.5*</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>12.9</td>
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<tr>
<td>First wash fluid</td>
<td>21</td>
<td>46.0</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>45.2</td>
</tr>
<tr>
<td>Once-washed cell**</td>
<td>21</td>
<td>4.9</td>
</tr>
<tr>
<td>suspension</td>
<td>80</td>
<td>6.7</td>
</tr>
<tr>
<td>Second wash fluid</td>
<td>21</td>
<td>41.1</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>32.8</td>
</tr>
<tr>
<td>Twice-washed** cell</td>
<td>21</td>
<td>3.4</td>
</tr>
<tr>
<td>suspension</td>
<td>80</td>
<td>7.3</td>
</tr>
</tbody>
</table>

*Based on average control value (25 replicates) of A_405 = 0.017 at 21 C and A_405 = 0.018 at 80 C.

**Equivalent bacterial concentrations in cells/ml: undiluted = 10^7; 10^-1 = 10^5; 10^-2 = 1.5; 10^-3 = 10^4 and 10^-4 = 10^5.

***Limit of visual detection.
Table 5. Effect of a 90 min heat treatment at 121 °C on detection by ELISA of *Erwinia carotovora* subsp. *atroseptica* antigens in undiluted distilled water cell wash fluids and washed cell suspensions (10^8 cells/ml) of fluids strain E193

<table>
<thead>
<tr>
<th>Treatment</th>
<th>20 °C</th>
<th>121 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^8</td>
<td>0</td>
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<tr>
<td>Normal cell suspension</td>
<td>1.159*</td>
<td>0.019</td>
</tr>
<tr>
<td>First wash fluid</td>
<td>1.321</td>
<td>0.051</td>
</tr>
<tr>
<td>Once-washed cell suspension</td>
<td>1.065</td>
<td>0.019</td>
</tr>
<tr>
<td>Second wash fluid</td>
<td>1.309</td>
<td>0.067</td>
</tr>
<tr>
<td>Twice-washed cell suspension</td>
<td>0.967</td>
<td>0.019</td>
</tr>
</tbody>
</table>

*Mean of five replicates.
When representative strains of the 24 *Erwinia carotovora* serogroups were tested, predictably the serogroup I strains gave the highest mean $A_{405}$ values (Table 6). However at the cell concentration used ($10^8$ cells/ml) strains of the three other subsp. *atroseptica* serogroups (XVIII, XX, XXII) plus three subsp. *carotovora* serogroups (III, IV, V) were also detected by the test system. Although the mean $A_{405}$ values obtained were only 10% of those obtained with the homologous strains, the absorbance ratios were greater than 2.0 and the wells containing these serogroups were visually detectable. With the exception of the serogroup II strain which was visually variable but spectrophotometrically negative, the remaining serogroups were similar to the buffer controls. Two other species often associated with natural *Erwinia* infections were also comparable to the controls. A 5 min heat treatment at 60°C generally increased the absorbance ratios for the subsp. *atroseptica* serogroups but decreased those of the subsp. *carotovora* serogroups at most cell concentrations. In spite of this selective enhancement, the limit of detection for the homologous strains remained at $10^6$ cells/ml. The subsp. *carotovora* strains, although still detectable by absorbance ratios at $10^7$ cells/ml, were variable or negative visually after heat treatment. The limit of detection of strains E368 and E555 corresponding to serogroups XX and XXII were unaffected by heating. Strain E17 (serogroup XVIII) was exceptional among subsp. *atroseptica* strains in that heating decreased the $A_{405}$ reading at $10^6$ cells/ml. As a consequence there was a 10-fold loss in sensitivity as determined by absorbance ratios.
Table 6. Absorbance ($A_{405}$), absorbance ratio (AR) and qualitative visual estimate of color development for $10^8$ cells/ml of strains representing 2 serogroups of Erwinia carotovora and other bacterial pathogens in an ELISA optimized for the detection of Erwinia carotovora subsp. atroseptica serogroup I

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Strain</th>
<th>Organism</th>
<th>Absorbance $A_{405}$</th>
<th>Visual estimate</th>
<th>Absorbance Ratio AR</th>
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<td>E68</td>
<td>atroseptica</td>
<td>1.515*</td>
<td>+</td>
<td>26.6</td>
</tr>
<tr>
<td></td>
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<td>1.588</td>
<td>+</td>
<td>27.9</td>
</tr>
<tr>
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<td>atroseptica</td>
<td>2.164</td>
<td>+</td>
<td>38.0</td>
</tr>
<tr>
<td></td>
<td>E331</td>
<td>atroseptica</td>
<td>1.608</td>
<td>+</td>
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<td>E95</td>
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<td>+</td>
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</tr>
<tr>
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<td>E14</td>
<td>carotovora</td>
<td>0.145</td>
<td>+</td>
<td>2.5</td>
</tr>
<tr>
<td>V</td>
<td>S26</td>
<td>carotovora</td>
<td>0.145</td>
<td>+</td>
<td>2.5</td>
</tr>
<tr>
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<td>S189</td>
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<td>-</td>
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<tr>
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<td>S68</td>
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<td>-</td>
<td>0.9</td>
</tr>
<tr>
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<td>S62</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>XIV</td>
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<td>-</td>
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<td>S23</td>
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<td>-</td>
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<tr>
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<td>XXI</td>
<td>E295</td>
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<td>-</td>
<td>0.9</td>
</tr>
<tr>
<td>XXII</td>
<td>E555</td>
<td>atroseptica</td>
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<td>Ps. marginalis</td>
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<td>C. sepedonicum</td>
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<td>-</td>
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</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>0.057</td>
<td>-</td>
<td>*</td>
</tr>
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</table>

*Mean of six replicates.
Table 7. Effect of a 5 min 60 C heat treatment on the detection by ELISA of known concentrations of selected strains of *Erwinia carotovora*

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Strain</th>
<th>Subsp.</th>
<th>Temp C</th>
<th>10^7 Absorbance (A_0.05)</th>
<th>Absorbance ratio (AR)</th>
<th>10^6 Absorbance (A_0.05)</th>
<th>Absorbance ratio (AR)</th>
<th>10^5 Absorbance (A_0.05)</th>
<th>Absorbance ratio (AR)</th>
<th>10^4 Absorbance (A_0.05)</th>
<th>Absorbance ratio (AR)</th>
<th>10^3 Absorbance (A_0.05)</th>
<th>Absorbance ratio (AR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>E193</td>
<td>atroseptica</td>
<td>21</td>
<td>1.708*</td>
<td>30.5</td>
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<td>14.1</td>
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<td>2.6</td>
<td></td>
<td>**</td>
<td>0.073</td>
<td>1.6</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>60</td>
<td>1.822</td>
<td>38.0</td>
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<td>0.313</td>
<td>6.1</td>
<td>0.095</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>S21</td>
<td>carotovora</td>
<td>21</td>
<td>0.072</td>
<td>1.3</td>
<td>0.070</td>
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<td>0.060</td>
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<td>0.184</td>
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<td>0.078</td>
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<td>0.056</td>
<td>1.0</td>
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<td>1.3</td>
<td>0.068</td>
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<td>E14</td>
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<td>0.088</td>
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<td>60</td>
<td>0.132</td>
<td>2.9</td>
<td>0.078</td>
<td>1.5</td>
<td>0.053</td>
<td>1.0</td>
<td>0.059</td>
<td>1.2</td>
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</tr>
<tr>
<td>V</td>
<td>S26</td>
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<td>21</td>
<td>0.131</td>
<td>2.3</td>
<td>0.073</td>
<td>1.6</td>
<td>0.047</td>
<td>0.8</td>
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<td>0.062</td>
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<tr>
<td>VIII</td>
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<td>1.0</td>
<td>0.049</td>
<td>1.1</td>
<td>0.054</td>
<td>0.9</td>
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<td>0.053</td>
<td>1.0</td>
<td>0.045</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>XVIII</td>
<td>E17</td>
<td>atroseptica</td>
<td>21</td>
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*Mean of six replicates

**Limit of visual detection
DISCUSSION

The detection of bacteria by ELISA, unlike viruses and γ-globulins, is complicated by the presence of several antigenic sites on bacteria, unless the antiserum is produced against a particular antigen. In this study, antiserum to glutaraldehyde-fixed whole cells was employed so that the specificity in ELISA could be directly compared with that in the double diffusion system previously described for Erwinia carotovora (DeBoer et al. 1979). Both water soluble antigen(s) as well as antigen(s) bound to the cell are apparently responsible for the reaction in ELISA. The fact that a small component of both the soluble and insoluble fractions was heat labile (Table 5) supports the conclusion that the primary antigen involved was LPS but that secondary labile protein antigens were also present.

The availability of these antigens appears to be at least partially influenced by growing conditions. Equal numbers of cells grown on different media were detected to different degrees by ELISA. The cells produced on Kmb which supported very large mucoid colonies, reacted poorly compared to those grown on media supporting small colonies. Whether this was due to physical masking of the reactive sites by the polysaccharides or to slight changes in cell envelope chemistry was not determined. The fact that washed cells were detected less well by ELISA than unwashed cells, tends to discount the first possibility. However, the enhanced detection after the cells were heated for periods as short as 3 min, suggests that a heat labile protein may be partially blocking the reactive sites. Kishinevsky and Bar-Joseph (1978) and Berger et al. (1979) have previously reported obtaining increased $A_{105}$ when Rhizobium cells were heated prior to assay. The latter authors speculated that the heating uncovers more reactive sites.
This ELISA system employing antiserum against glutaraldehyde fixed whole cells of Eca strain E82 was quite specific. No cross reactions were observed with P. marginalis, and C. sepedonicum even at high \(10^8\) cells/ml concentrations. However, at high cell concentrations cross reactions were observed with a few strains of Ecc belonging to serogroups II, III, IV and V. Similarly, strains falling into the other 3 serogroups which are biochemically subsp. atroseptica also reacted. Vruggink (1978) also reported cross reactions with some strains although the serogroups were unknown. Such a result is not surprising because DeBoer (1980) has reported that serogroups I, III, V and XVIII share a common heat-labile flagellar antigen, and that the cell wall antigens of serogroups I, II and XVIII are related. Heating the cells prior to assay did reduce but not completely eliminate the reactions of strains in serogroups III and V at \(10^7\) cells/ml, as both AR > 2.0 and positive visual estimates were recorded. Strain E17 belonging to serogroup XVIII was exceptional among the subsp. atroseptica strains in that heat treatment at \(10^6\) cells/ml resulted in a 10-fold loss of sensitivity. The possibility exists that as the limits of sensitivity are approached, removal of the common labile flagellar antigen may have been sufficient to result in a negative reaction. The fact that the somatic antigens of serogroups I and XVIII are related explains why higher absorbance ratios were obtained than with the subsp. carotovora strains except those belonging to serogroup II. The latter also shares a common somatic antigen with serogroup I and might have been expected to have a higher value. Why the strain of this later serogroup did not react more strongly after heat treatment is unknown. Heat treatment markedly increased the absorbance ratio of the homologous antigen while only slightly increasing those of the remaining two subsp. atroseptica serogroups. Thus in the range of \(10^6\) and \(10^5\) cells/ml, this ELISA was serogroup specific. Heat
treatment of the samples increased the absorbance ratios but did not change the pattern of specificity. At higher cell concentrations the possibility of cross reactions with serogroup III which is most commonly found on potato tubers (DeBoer et al. 1979), and the other subsp. atroseptica serogroups (XVIII, XX and XXII) certainly exists. Depending upon the purpose of the detection work, being able to detect these important serogroups might be desirable.

The potential relationship between serogroup IV and I detected as a cross reaction in this study was not detected by DeBoer (1980). However, this observation is not surprising because due to the sensitivity of the ELISA system, cross reactions and relationships not detectable by other means have been previously detected by ELISA (Carlsson et al. 1976).

Even after standardized washing and selective enhancement by heat treatment, the maximum limit of sensitivity in this ELISA for Erwinia carotovora subsp. atroseptica (serogroup I) is $10^5$ cells/ml based on both AR > 2.0 and positive visual color development. This compares favorably with the reported level of sensitivity of $10^6 - 10^7$ cells/ml for Pseudomonas phaseolicola (Weaver and Guthrie 1978). Stevens and Tsiantos (1979) claimed $10^3$ cells/ml as the limit of detection for Corynebacterium michiganense in buffer suspensions. However, based on their Figure 2, a more likely limit of detection is $10^5 - 10^6$ cells/ml. Because the curve was essentially at the same level from $10^3$ to $10^5$ cells/ml, it is difficult to understand how they can claim sensitivity at the lower level. Cother and Vruggink (1980) working with Erwinia carotovora subsp. atroseptica reported, as unpublished data, that the limit of detection in their system was $10^4$ cells/ml, but no data have been published to confirm this figure. The level of $10^5$ cells/ml is at or above the limit of sensitivity of immunofluorescence reported by Slack et al. (10 to $10^2$ cells/
ml) 1979) and Allan and Kelman (10^5 cells/ml) (1977). These data confirm Vruggink's (1978) report that both IFAS and ELISA gave comparable results with Eca. The optimized ELISA technique as used in this study compared to immunofluorescence for detection of Eca offers only a reduction in operator fatigue, and an objective means of assessment but no greater sensitivity nor a more rapid test.

The fact that the primary antigen detected in this system is water soluble provides the opportunity for tests based on cell suspensions or cell extracts. The high absorbance ratios associated with cell wash fluids in Tables 3 and 4 require cautious interpretation. In these experiments suspensions containing very high concentrations of cells (≈ 10^{10}) were washed to maximize the presence of soluble antigens. Thus, although the bacterial suspensions that were used were standardized to 10^8 cells/ml, the washing fluids used did not correspond to the washing fluids from a 10^8 cells/ml suspension but rather to the original more concentrated suspension. It remains to be determined whether similar data would be obtained from the wash fluids of lower cell concentrations, and whether a test based on just the soluble antigen is possible.

The ELISA for Eca was found to have serogroup specificity at the medium to low concentrations (10^5 - 10^6 cells/ml) at which it would be most likely used. The obstacle to its widespread use is sensitivity. In spite of employing optimized conditions of coating and conjugate concentrations, washing and selective enhancement by heat, variability in the plates precludes lowering the limit of sensitivity beyond 10^5 cells/ml. Until or unless this variability with Erwinia carotovora subsp. atroseptica can be eliminated or reduced, ELISA seems to have little potential in routine surveys for detecting latent blackleg infection in certified seed potatoes.
LITERATURE CITED


VOLLER, A., D.E. BIDWELL and A. BARTLETT. 1977. The enzyme-linked immunosor­

VRUGGINK, H. 1978. Enzyme-linked immunosorbent assay (ELISA) in the sero-

DISCUSSION

This enzyme-linked immunosorbent assay (ELISA) model system, employing *Erwinia carotovora* subsp. *atroseptica*, was based on criteria seldom used simultaneously. The requirement for both absorbance ratios \( \geq 2.0 \) and a positive visual estimate, following a 30-min reaction time, imposed more stringent requirements for a positive test than are normally used. By requiring both criteria, only strong and rapid reactions which had visibly negative controls were considered. At high bacterial concentrations \((10^6 - 10^7 \text{ cells/ml})\) there was consistency of the results with both criteria, while at lower concentrations \((10^4 - 10^5 \text{ cells/ml})\) some differences could be observed, and the percent variability remained high. These discrepancies at low bacterial concentrations were attributed mainly to the background. This variable background influenced the AR \( \geq 2.0 \) and biased the conclusions about detection at the limit of sensitivity.

The use of a standardized washing procedure removed some of the variability by removing some of the background. Regardless of the wash solution employed, the use of a controlled pressure device was always much better than a wash bottle. The addition of Tween-20 in the washing solution should be avoided unless a plate reader with dual wavelength is available because it leaves a film at the bottom of plates, which interfered with the readings. While the presence of Tween-20 did not increase the visual background, lower limits of sensitivity were obtained both visually and on an absorbance ratio \( \geq 2.0 \) basis, which might be the result of the detergent effect of Tween-20. The use of distilled water was as good as phosphate buffered saline, and more practical with the controlled pressure system adopted. It was also important that the selection of the coating \( \gamma \)-globulin concentration and the enzyme-\( \gamma \)-
globulin conjugate dilution combination be done under standardized washing conditions, because excessive washing of the various components, resulted in a severe decrease of sensitivity.

The well to well variability observed within a plate, as well as the plate to plate variability were primarily responsible for the low limit of sensitivity of the technique. These sources of variability were maintained despite an optimized coating-conjugate combination, and a standardized washing procedure. This type of variability caused problems especially at low coating concentration and high conjugate dilutions, where background variations made it hard to determine what was positive. Moreover, this variability within or between plates might possibly mask other potential sources of variability. Among these, the buffers used for the samples or the conjugate dilutions showed differences but were not significantly different from the one employed in virus work. Whether the requirements for \textit{in vitro} work employing only pure cultures, and those for \textit{in vivo} work involving plant sap might be different, remains to be determined. The different results obtained between buffers, and the fact that the main effect was related to the conjugate, suggested that the requirements for binding the bacterial cells to the coating $\gamma$-globulin could be different from those for binding of the enzyme-$\gamma$-globulin conjugate to the bacterial cells. None of the variability observed could be related to pipetting errors. It is possible that improved plates with less inherent variability might permit uncovering sources of variation actually masked in this work.

At this point, the ELISA technique as employed for \textit{Erwinia carotovora} subsp. \textit{atroseptica} does not offer real advantages over immunofluorescence with regard to both specificity and sensitivity. Although heat treatment of the cells showed a selective enhancement, the sensitivity of the technique
could not be lowered below \(10^5\) cells/ml. Furthermore, the serogroup specificity at lower bacterial concentrations (\(10^6\) cells/ml) was comparable with or without heat treatment of the cells, to that shown by immunodiffusion. The data obtained by heat treatment, cell washing, and the observed specificity support the idea that the lipopolysaccharide (LPS) is probably the major antigen in this test. Since the availability of the antigen was a function of the growth medium, it will be interesting to know how cells from naturally-infected plants would compare. Because LPS is partly soluble in water, further use of this fact should be made in future trials.

The work reported here, obviously suffers the disadvantage that only one antiserum was used throughout. However, encouraging and valuable information was obtained. Whether this is representative, and whether antisera to other antigens might be better remain to be investigated.

Until variability is eliminated and sensitivity increased, there will be little incentive to use the double sandwich ELISA technique with plant sap where reduction in sensitivity is likely. At this point ELISA seems to have little potential in routine surveys for detecting latent blackleg infection in certified seed potatoes.
SUMMARY

1. The combination of 2.0 μg/ml coating γ-globulin and 1:400 enzyme-γ-globulin conjugate dilution were determined as optimum for the Erwinia carotovora subsp. atroseptica system with this particular antiserum and type of microtitration plates.

2. The washing procedure was a variable affecting detection which had to be standardized. A controlled pressure-washing system employing distilled water, and two 15-sec washes at 34.48 kPa (5 psi), with 180° rotation of the plate between each wash was adopted.

3. The well to well variability in the microtitration plates was not exclusive to the outside rows and was shown to decrease with time of reaction.

4. The buffer solutions employed for dilution of samples and conjugate influenced the A₄₀⁵ values. The conjugate buffer had a greater effect than the sample buffer.

5. The complete buffer used in virus work (PBS + 0.05% Tween-20 + 2.0% PVP + 0.2% egg albumin) was a suitable choice for both sample and conjugate preparation.

6. The limit of detection of E. carotovora subsp. atroseptica was 10⁵ - 10⁶ cells/ml.

7. Pipetting errors of 5% in coating, sample and/or conjugate did not reproduce the observed variability.

8. Heat treatment of the cells at 60 C for 3 - 6 min enhanced A₄₀⁵ values but the level of sensitivity following a heat treatment was 10⁵ cells/ml.

9. The medium upon which cells were grown affected the A₄₀⁵ values but was not proportional to the amount of growth observed.
10. Washing bacterial cells in distilled water resulted in the detection by ELISA of soluble antigens in both the wash fluids and the resuspended cell suspensions.

11. Heat-labile and heat-stable antigens were present in both the wash fluids and the resuspended cells.

12. Specificity of the *E. carotovora* subsp. *atroseptica* serogroup I ELISA was maintained at cell concentrations of less than $10^5$ cells/ml.

13. Serological cross reactions among *E. carotovora* serogroups at high cell concentrations (> $10^7$ cells/ml) were confirmed by ELISA.
SUPPLEMENTARY LITERATURE CITED


