Antibody Sandwich Enzyme-Linked Immunosorbent Assay

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ABSTRACT

Polyclonal antibodies raised against live cells of *Pseudomonas fluorescens* AH-70 have been used in a double antibody sandwich ELISA technique for the detection of *P. fluorescens* and related psychrotrophic bacteria in refrigerated milk. These antibodies, bound to the wells of a microtiter plate, were used to capture the microorganisms on milk samples. Further immunorecognition of the captured antigens was attained with the same antibodies conjugated to biotin. ExtrAvidin®-peroxidase was used to detect the biotinylated antibodies bound to their specific antigens. Subsequent enzymic conversion of substrate showed clear differences in absorbance after assay of milk samples containing *P. fluorescens* strains of different origin and related psychrotrophic microorganisms. The detection threshold for the sandwich ELISA assay developed in this work was 10^5 cfu/ml.

(**Key words**: refrigeration of milk, *Pseudomonas fluorescens*, psychrotrophic bacteria, enzyme-linked immunosorbent assay)

Abbreviation key: **ALC** = polyclonal antibodies raised against live cells of *Pseudomonas fluorescens* AH-70, **PBST** = PBS containing 5% Tween 20.

INTRODUCTION

Use of refrigeration to prolong the storage life of milk on the farm, in the dairy plant, during marketing, and in the home of consumers has increased the importance of the psychrotrophic bacteria (18). Many of these microorganisms produce heat-resistant proteinases and lipases that may reduce the quality and shelf-life of heat-treated milk and dairy products made from contaminated milk. Proteinases induce bitterness and gelation of UHT-sterilized milk (7), and lipases cause flavor defects because of fat breakdown in cream, butter, and cheese (6, 15, 24).

A correlation exists between the number of psychrotrophic bacteria and the enzyme activity in milk samples (1). Thus, managers of dairy factories need to know the initial psychrotrophic bacterial count of the incoming raw milk to determine the maximum length of refrigerated storage that is not harmful to cheese yield or milk quality. Rapid and reliable tests to assess the bacterial content of refrigerated milk are thus needed (29).

Standard methods for determination of psychrotrophic bacterial counts require incubation of plates at 7°C for 10 d. Under these conditions, milk often spoils before information on product quality is available. In an attempt to reduce the time required for psychrotrophic enumeration, many investigators have proposed different methods to overcome the disadvantages inherent in traditional plate count methods, including fluorescence microscopy (21), adenosine triphosphate photometry (3), the aminopeptidase method (20), the Limulus test (16), and the ELISA (8, 9, 14).

Among the methods used for the rapid detection and counting of bacteria in foods, immunochemical techniques are promising because of their sensitivity and rapidity (22). The ELISA has become the most commonly used form of immunoassay in food analysis because of its simplicity, specificity, and ability for use

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as a rapid screening method for a large number of samples (5).

Based on several reports (13, 19, 27, 28) describing the use of live bacterial cells in immunization schedules to generate antibodies suitable for immunological detection of microorganisms, we report in this study the use of polyclonal antibodies raised against the live cells of *Pseudomonas fluorescens* AH-70 (**ALC**) to detect and to quantify the presence of *P. fluorescens* and related psychrotrophic bacteria in refrigerated raw milk.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Pseudomonas fluorescens strains DC-5, DC-7, and NT-19 from meat origin were supplied by B. G. Shaw, Food Research Institute, Bristol, England. Strains B-52 and AR-11 from milk origin were, respectively, from R. C. McKellar, Food Research Centre, Ottawa, Ontario, Canada and from B. A. Law, National Institute for Research in Dairying, Shinfield, Reading, England. *Pseudomonas fluorescens* strain AH-70 from Spanish refrigerated milk was previously isolated and identified in our laboratory (2). The microorganisms were usually grown at 24°C in a medium containing 1% tryptone, 1% yeast extract, and .5% NaCl (Oxoid, Unipath, Basingstoke, England).

Pseudomonas aeruginosa, Pseudomonas fragi, Flavobacterium odoratum, Achromobacter cycloclastes, Acinetobacter calcoaceticus, Alcaligenes denitrificans, Klebsiella sp., and *Enterobacter aerogenes* were from the Spanish Type Culture Collection, Departamento de Microbiología, Facultad de Ciencias Biológicas, Valencia, España.

Immunization Schedule

The ALC were raised in male New Zealand white rabbits. Immunization was by subcutaneous injection of 10^9 cfu of live cells of *P. fluorescens* AH-70 emulsified in .5 ml of Freund's incomplete adjuvant (Difco, Detroit, MI) and .5 ml of deionized distilled water. This volume was distributed among injections at multiple sites along the back of each rabbit. Booster doses (n = 17) made as described were injected subcutaneously every 7 d. After 4 mo, the rabbits were bled, and the blood was allowed to clot for 1 h at 20°C. Serum was collected after centrifugation at 2000 × *g* for 10 min. Samples of crude antiserum (1 ml) were stored frozen at –20°C.

The Ig fractions containing the ALC were recovered from the crude antiserum by ammonium sulfate precipitation (10). Samples (10 ml) of the crude antisera were centrifuged at 3000 × *g* for 30 min, the pellet was discarded, and 10 ml of a saturated solution of ammonium sulfate (pH 7.4) were added to the supernatant fluid. After overnight incubation at 4°C, the precipitate was collected by centrifugation at 3000 × *g* for 10 min, dissolved in 5 ml of PBS (136 m*M* NaCl, 1.4 m*M* KH_2PO_4, 8.09 m*M* $Na_2HPO_4 \cdot 12H_2O$, and 2.6 m*M* KCl; pH 7.2), and dialyzed overnight against the same buffer and lyophilized.

Biotinylated Antibodies

Biotinamidocaproate N-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, MO), dissolved at 1 mg/ml in dimethylsulfoxide (Merck, Darmstaat, Germany), was added at a molar excess of 50 to the ALC dissolved in PBS (1 mg/ml) and incubated for 2 h at 20°C (4). Unbound reagent was separated from the conjugated antibodies by dialysis overnight at 4°C against PBS. Samples of biotinylated ALC (.1 ml) were stored at –20°C until use.

Milk Samples

Reference samples of UHT-treated milk were inoculated with pure cultures of *P. fluorescens* strains AH-70, AR-11, B-52, DC-5, DC-7, and NT-19 to obtain bacterial counts of 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 cfu/ml. When indicated, inoculated milk samples were subjected to pulses of sonication of 15 × 10 s (Heat Systems Ultrasonic Inc., Plainview, NY) at 50 kHz with 15-s intervals for cooling. The temperature of the cell suspension was kept at 0°C in a jacketed glass vessel connected to an ice bath throughout the ultrasonic process.

The raw milk samples were obtained by aseptic milking of healthy cows from a local dairy farm and then maintained at 4°C. The samples were examined every 24 h for psychrotrophic bacterial counts by incubation of plate count agar plates (Difco) at 17°C for

17 h and then at 7°C for 72 h (23) and by the double antibody sandwich ELISA procedure.

Double Antibody Sandwich ELISA Procedure

Flat-bottomed micro-ELISA plates (Costar, Cambridge, MA) were filled with .1 ml of the captured ALC (1 mg/ml) diluted 1:1000 (vol/vol) in PBS, pH 7.2, and incubated for 1 h at 37°C. The wells were washed five times with **PBST** (PBS containing 5% Tween 20, vol/vol) and coated with .2 ml of .1% gelatin in PBS for 1 h at 37°C. After five washes with PBST, .1 ml of a milk sample was added to the wells, and the plates were incubated on a plate shaker (LKB, Pharmacia, Uppsala, Sweden) for 1 h at 20°C. Following another washing with PBST, biotinylated ALC (1 mg/ml), diluted 1:1000 (vol/vol) in PBST, were added to the wells, and the plates were incubated with shaking for 1 h at 20°C. After five more washes with PBST to remove free antibodies, .1 ml of ExtrAvidin®-peroxidase (Sigma Chemical Co.), diluted 1:3000 (vol/vol) in PBST, was added to the wells, and plates were incubated with shaking for 1 h at 20°C. Wells were washed five more times with distilled water before addition of .15 ml of the substrate solution consisting of .6 mg/ml of 2,2′-azinobis-3-ethyl-benzthiazoline sulfonic acid (Sigma Chemical Co.) in citric acid-phosphate buffer (pH 3.9) with H_2O_2 (30%, wt/wt; .2 μl/ml of buffer). After a 10-min incubation, the reaction was stopped by addition of .05 ml of sodium fluoride (2% in distilled water) to each well. The green color developed by conversion of substrate was measured at 405 nm with a spectrophotometer (Titertek Multiskan Plus; Flow Laboratories, McLean, VA).

RESULTS

This double antibody sandwich ELISA used ALC. An exhaustive checkerboard titration of all components of the assay showed that conditions for the sandwich ELISA were optimal using the captured ALC and the biotinylated ALC diluted 1:1000 (vol/vol) and the ExtrAvidin®-peroxidase (Sigma Chemical Co.) conjugate diluted 1:3000 (vol/vol).

The results of the double antibody sandwich ELISA developed in this work (Figure 1) showed the absorbance from samples of commercially UHT-treated bovine milk inoculated with a pure culture of *P. fluorescens* strains AH-70, AR-11, B-52, DC-5, DC-7, and NT-19. The ALC recognized strongly the presence of *P. fluorescens* AH-70 in milk samples, and, over a limited range of concentrations of bacterial cells (10^3 to 10^8 cfu/ml), absorbance increased with the bacterial population, and detection threshold was 10^4 cfu/ml. However, specificity of the ALC to recognize other tested *P. fluorescens* strains was slightly reduced, and the detection threshold for most of the bacterial populations decreased to 10^5 cfu/ml. To increase the sensitivity of the assay, an experiment was performed to measure the response of the ALC against sonicated milk samples inoculated with the same *P. fluorescens* strains. Figure 2 shows that the absorbance from bacterial populations disrupted by sonication was higher than that with intact cells and that the ALC recognized all *Pseudomonas* strains tested with a detection threshold of 10^4 cfu/ml.

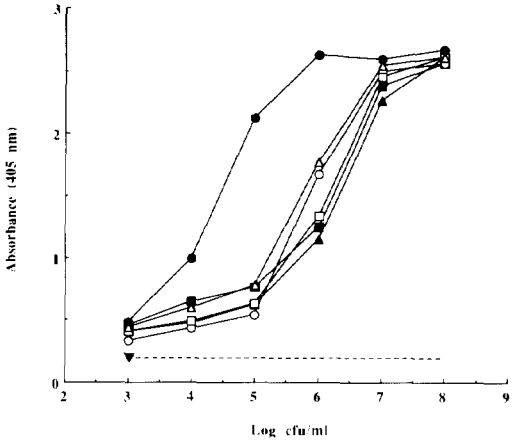

Figure 1. Double antibody sandwich ELISA results for samples of UHT-treated milk inoculated with pure cultures of *Pseudomonas fluorescens* strains AH-70 (●), AR-11 (■), B-52 (▲), DC-5 (○), DC-7 (□), and NT-19 (△). Control sample is UHT milk without inoculum (▼). The captured anti-live cells antibodies and the detection biotinylated antibodies were diluted 1:1000 (vol/vol). The ExtrAvidin®-peroxidase was diluted 1:3000 (vol/vol).

The specificity of the ALC was evaluated against a range of psychrotrophic microorganisms, including *P. aeruginosa, P. fragi, F. odoratum, Achr. cycloclastes, Acin. calcoaceticus, Alc. denitrificans, Klebsiella* sp., and *E.*

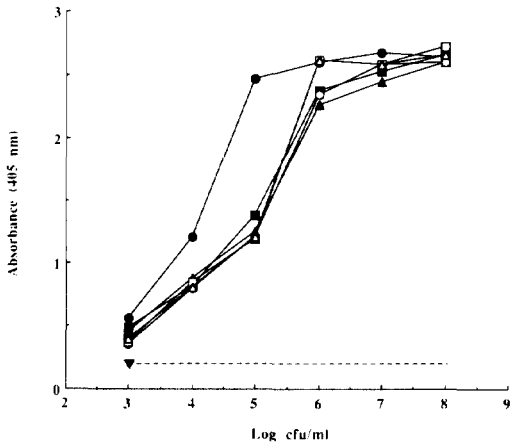

Figure 2. Double antibody sandwich ELISA results for samples of sonicated UHT-treated milk inoculated with pure cultures of *Pseudomonas fluorescens* strains AH-70 (●), AR-11 (■), B-52 (▲), DC-5 (○), DC-7 (□), and NT-19 (△). Control sample was UHT milk without inoculum (▼). The captured anti-live cells antibodies and the detection biotinylated antibodies were diluted 1:1000 (vol/vol). The ExtrAvidin®-peroxidase was diluted 1:3000 (vol/vol).

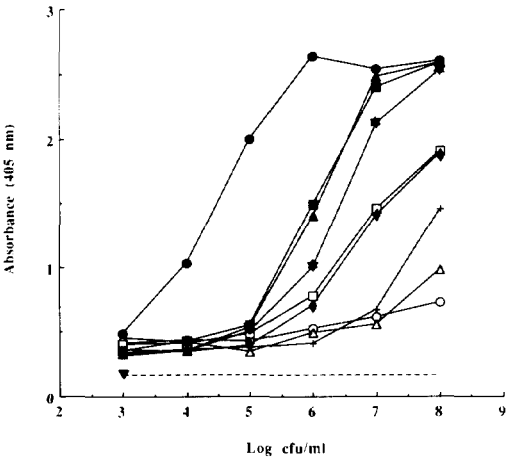

Figure 3. Double antibody sandwich ELISA results for samples of UHT-treated milk inoculated with pure cultures of *Pseudomonas fluorescens* AH-70 (●), *Pseudomonas aeruginosa* (■), *Pseudomonas fragi* (▲), *Flavobacterium odoratum* (○), *Achromobacter cycloclastes* (□), *Acinetobacter calcoaceticus* (△), *Alcaligenes denitrificans* (♦), *Klebsiella* sp. (★), and *Enterobacter aerogenes* (+). Control sample was UHT-treated milk without inoculum (▼). The captured anti-live cells antibodies and the detection biotinylated antibodies were diluted 1:1000 (vol/vol). The ExtrAvidin®-peroxidase was diluted 1:3000 (vol/vol).

aerogenes. The results obtained (Figure 3) showed that the ALC recognized the strains of *P. aeruginosa* and *P. fragi* with the same specificity as the *P. fluorescens* strains previously analyzed (Figure 1). However, the specificity of the ALC to recognize other psychrotrophic bacteria tested was reduced.

The sandwich ELISA was also used to detect and to quantify the psychrotrophic bacterial content of raw milk samples maintained at 4°C. Figure 4 shows the relationship between bacterial psychrotrophic counts and absorbance at 405 nm. In these sonicated milk samples, absorbance was related to psychrotrophic bacterial counts (B) in the range 10^5 to 10^9 cfu/ml by the equation: log absorbance = −2.4105 + .3519 log (B) (r = .98; P = .0001). These milk samples, containing bacterial counts from 10^5 to 10^9 cfu/ml, may be used as a standard when the presence of psychrotrophic microorganisms is quantified in stored refrigerated milk. This standard curve is specific for the ALC developed herein.

DISCUSSION

The outer membrane of *P. aeruginosa*, like all Gram-negative bacteria, is a bilayer containing lipopolysaccharides, phospholipids, and a few major proteins (12). Protein F is the major porin protein of the outer membrane, exposed on the cell surface and conserved and

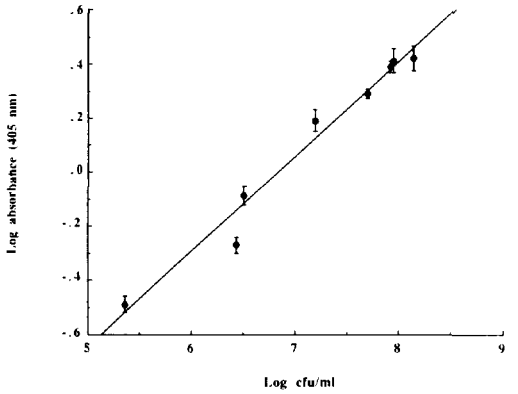

Figure 4. Relationship between psychrotrophic plate counts and the absorbance values obtained by the sandwich ELISA in milk samples stored at 4°C. Bars represent standard deviation of six assays from two independent milk samples.

antigenically related in all tested *P. aeruginosa* strains (17, 30). Based on this information, we previously reported the use of polyclonal antibodies against the protein F from *P. fluorescens* AH-70 for detection of *P. fluorescens* and related psychrotrophic bacteria in refrigerated milk and meat (8, 9). The results revealed that the anti-protein F antibodies of *P. fluorescens* AH-70 recognized strongly the presence of this strain in milk samples, but their capacity to recognize other *P. fluorescens* strains was significantly reduced. Furthermore, the lower detection threshold for the ELISA assay developed was 10^6 cfu/ml. To make the ELISA technique more sensitive, we hypothesized that ALC could be used to detect and to quantify the presence of *P. fluorescens* and related psychrotrophic microorganisms in refrigerated milk.

Several reports (13, 19, 27, 28) have demonstrated that antibodies produced against live and inactivated bacteria may be suitable for immunological detection of microorganisms. However, Stya et al. (25) have suggested that inactivation treatments can expose intracellular antigens or alter the structural configuration of the membrane antigens. Thus, antibodies raised to inactivated cells may fail to recognize the configuration of live bacteria (13, 25). According to these observations, we raised polyclonal antibodies against the live cells of *P. fluorescens* AH-70.

The double antibody sandwich ELISA described herein uses ALC to capture the microorganisms in milk samples. Immunorecognition was enhanced with the same antibodies conjugated to biotin. The biotinylated ALC bound to the bacterial cells were detected with ExtrAvidin®-peroxidase (Sigma Chemical Co.), a commercially available modified avidin reagent conjugated to horseradish peroxidase. This reagent combines the high specific activity of avidin with the low background response associated with streptavidin (11).

Figure 1 shows that ALC recognized strongly the presence of this strain in milk samples, but their specificity to recognize the presence of other *P. fluorescens* strains was slightly reduced. This observation may be explained by hypothesizing that the cell surface antigens from the strain AH-70 contain epitopes not present in other analyzed *Pseudomonas* sp. The sensitivity of the ELISA increased when milk samples inoculated with *Pseudomonas* strains were sonicated (Figure 2). Sonication of milk samples may contribute to the exposure of a larger number of epitopes to the antibodies.

Previous work carried out on the detection of *P. fluorescens* in refrigerated milk (8) revealed that the anti-protein F antibodies cross-reacted only with *Pseudomonas* sp., suggesting that protein F from *P. fluorescens* was specific to the genus. The results of the present study show that the ALC recognized the strains of *P. aeruginosa* and *P. fragi* with the same specificity of the *P. fluorescens* strains previously analyzed. However, the specificity of the ALC to recognize other psychrotrophic bacteria tested was reduced (Figure 3). This difference may be explained because the antiserum was raised against a complex mosaic of cell envelope antigens, which could present common surface epitopes in the different psychrotrophic bacteria.

Although the lower detection threshold for the ELISA assay is 10^5 cfu/ml (Figure 4), that of the Limulus test is 10^3 to 10^4 cfu/ml for a positive response. The Limulus test probably has the highest potential to assess the bacterial content of refrigerated milk (16), but the high cost of the amoebocyte lysate makes it very expensive for routine purposes (26). An advantage of the double antibody sandwich ELISA developed herein is that it does not require previous isolation or concentration of bacterial cells from milk samples. Moreover, because of its versatility, simplicity, and speed, this test can be used to screen stored refrigerated milk rapidly for the detection of high concentrations of psychrotrophic bacterial cells.

CONCLUSIONS

The ALC can be used in a double antibody sandwich ELISA technique for the detection of *P. fluorescens* strains from different origin and related psychrotrophic bacteria in refrigerated raw milk in the range of 10^5 to 10^9 cfu/ml.

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