JOURNAL OF CLINICAL MICROBIOLOGY, July 1982, p. 141–144
0095-1137/82/070141-04$02.00/0

Vol. 16, No. 1

Whole-Bacterial Cell Enzyme-Linked Immunosorbent Assay for *Streptococcus sanguis* Fimbrial Antigens

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Received 11 January 1982/Accepted 19 April 1982

A whole-bacterial cell enzyme-linked immunosorbent assay (bactELISA) was developed for detecting fimbrial antigens on *Streptococcus sanguis*. In this assay, *S. sanguis* cells were directly adhered to polystyrene or polyvinyl via drying. Use of the assay indicated that consistently high and uniform optical densities could be obtained from well to well. In addition, radioactive assaying indicated increased adsorbance to the polystyrene wells over polyvinyl, suggesting that polystyrene may prove superior in the gram-positive bactELISA. Use of the bactELISA may prove valuable to both the clinical and research laboratory involved in the study of bacterial cell surface components or in the evaluation of antisera directed against bacterial antigens, which are difficult to prepare as purified derivatives.

The enzyme-linked immunosorbent assay (ELISA) is rapidly becoming a central tool in both clinical and research laboratories (1–3, 7). The assay is simple and rapid, and since it rivals the radioimmunoassay in sensitivity, the lack of radioactive waste products makes it an ideal test system for both antigens and antibodies (9). To date, the assay has been limited by the necessity for purified or partially purified soluble antigen or antibody as the primary adsorbant to the solid-phase surface, although recently the adherence of whole mammalian cells has been successfully performed in the cell ELISA (CELISA) (2, 4; R. Morris, P. Thomas, and R. Hong, Abstr. Annu. Meet. Am. Assoc. Clin. Histocompatibility Testing, 1981, A61, p. 50). (The term CELISA has also been used by Pronovost et al. [8] to describe a chemiluminescent ELISA.) Only two laboratories have reported using whole bacterial cells in the ELISA. Cummings et al. (3) have used the ELISA for detection of cell wall carbohydrates in the grouping of beta-hemolytic streptococci adsorbed to microtiter wells, whereas Ison et al. (5) have used plates coated with *Neisseria gonorrhoeae* to detect antibodies in patient sera. Antibody presence was correlated with the presence or absence of a disease state.

The advantages of a whole-bacterial cell ELISA (bactELISA) are numerous. Coating with the whole cell is as simple and rapid as with soluble purified antigen. The wells can be coated for 1 h or overnight, depending on convenience. Preparation of the bacteria involves simple washing rather than laborious or expensive purification procedures and can be carried out by any laboratory. In addition, use of whole cells obviates the necessity of antigen purification in cases in which techniques are not available for the antigen of interest and allows the use of the sensitive ELISA over methods such as slide agglutination.

This paper reports the development of a bactELISA with whole *Streptococcus sanguis* cells. In our laboratory, the bactELISA is being used as a screen for traditional and monoclonal fimbrial antibody under production in experimental animals, as well as the primary tool for serotyping of *S. sanguis* fimbriae isolated from dental plaque samples. Although earlier investigators have used ELISA as a serological test for the study of fimbriae (1), they found it necessary to first remove the structures from the cell before attempting adherence to the well surface. With *S. sanguis*, this removal has proved to be a difficult task; hence, the bactELISA was developed (our laboratory; data not shown).

MATERIALS AND METHODS

Bacteria. *Streptococcus sanguis* FW213 (obtained from Roger Cole, National Institutes of Health) and *S. sanguis* JL7 (an isogenic nonadherent mutant of FW213 isolated in this laboratory and possessing only one of the three fimbrial types found on FW213 [P. Fives-Taylor, *in* D. Schlessinger, ed., *Microbiology—1982*, in press]) were used in this study. Both strains were kept frozen at −70°C, with aliquots removed weekly.

Organisms to be used in the bactELISA were plated for confluent growth on a tryptose blood agar base (Difco Laboratories, Detroit, Mich.), to which 5% defibrinated sheep blood had been added, and grown for 12 to 15 h at 37°C in 5% CO_2. The organisms were removed from the plate and suspended in 40 ml of Todd-Hewitt broth (Difco), incubated at 37°C with

gentle agitation, and assayed turbidimetrically until an optical density equivalent to 5.5×10^8 bacteria per ml was reached. The cells were washed three times in phosphate-buffered saline (0.05 M, pH 7.4) and suspended to a final concentration of 2.6×10^8 bacteria per ml in carbonate coating buffer (0.05 M, pH 9.6).

Antisera. Antisera to *S. sanguis* FW213 were produced in rabbits by a single subcutaneous injection of 10^9 live organisms in 0.85% saline. Rabbits were bled 2 months after injection. To remove nonfimbrial antibody, 1-ml samples of antisera were adsorbed with 0.1 ml of packed *S. sanguis* JL7. Each 0.1-ml sample of cells required 40 ml of JL7 prepared as described above for *S. sanguis* FW213. The adsorptions were performed at 4 and 37°C for 0.5-h periods (two adsorptions at 4°C followed by two at 37°C; the cycle was repeated for a total of eight adsorptions).

Preparation of ELISA plates. Ninety-six-well, flat-bottomed, processed microtitration polystyrene plates (Linbro-Titerek, Flow Laboratories, Inc., Rockville, Md.) were used. Each well received 150 μl of the appropriate bacterial suspension in carbonate coating buffer (equivalent to 4.5×10^7 bacteria per well). This concentration was chosen after microscopic examination (with an Olympus inverted microscope) of wells coated with 10^5, 10^6, 10^7, 10^8, and 10^9 bacteria per well revealed even and homogeneous coverage at suspensions of both 10^7 and 10^8 bacteria per well. After overnight adsorption at 4°C, the plates were dried with a hair dryer and washed three times with phosphate-buffered saline (0.05 M, pH 7.4) containing 0.02% sodium azide and 0.05% (vol/vol) Tween 20 (PBST). Bacterial adherence was confirmed by scanning with an inverted microscope at the beginning and end of each assay. Nearly confluent coverage of all wells (with only intermittent areas of nonadherence) was considered satisfactory.

ELISA technique. The ELISA used was based on that of Voller et al. (10). A 1:200 dilution of antiserum in PBST was made, and 200 μl was added to the bacteria-coated wells and incubated for 2 h at room temperature. The plate was then washed three times with PBST. Two hundred microliters of affinity-purified goat anti-rabbit immunoglobulin G (IgG) (H + L) conjugated with alkaline phosphatase (Dynatech Diagnostics, Windham, Maine) was added, and the plate was incubated for 3 h at room temperature. After the plate was washed three additional times in PBST, 200 μl of alkaline phosphatase substrate solution containing 1 mg of *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) per ml in 10% diethenolamine buffer (pH 9.6) was added to each well. The plate was incubated for 15 min at room temperature. The color reaction was stopped by adding 50 μl of 3 N NaOH, and the optical density was read at 410 nm with either a Microelisa Minireader Mr590 (Dynatech Laboratories, Alexandria, Va.) or a Chromoscan EIA Reader (Bio-Tek Instruments, Burlington, Vt.). Controls included the following: (i) wells with carbonate coating buffer and no bacteria, (ii) wells with PBST in place of antiserum, (iii) wells with no conjugate, and (iv) wells with normal rabbit serum in place of immune serum.

Tritium labeling of bacteria. To assess the amount of binding to the polystyrene well surface and to the surface of a 96-well, flat-bottomed, polyvinyl plate (Flexible Assay Plate; Falcon Plastics, Oxnard, Calif.), *S. sanguis* FW213 and JL7 were grown in Todd-Hewitt broth containing [^{3}H]thymidine at a final concentration of 2 μCi/ml. After the bacteria were adsorbed to the microtiter plate and washed nine times with PBST, the wells were separated, placed in 5 ml of Aquasol-2 (New England Nuclear Corp., Boston, Mass.) and counted in an LS7500 scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.).

RESULTS

Detection of antibodies to *S. sanguis* FW213. When tested with the methodology described above, antisera directed against *S. sanguis* FW213 gave consistently high and uniform optical densities (Table 1). Extensive adsorption with the nonadherent mutant JL7 removed 84% of the antibodies which reacted to antigens held in common between the parent organism and the isogenic mutant. Reaction of a preimmunization serum with either organism revealed no activity above the blank (wells with no substrate). All control wells were negative for color development.

Assay for bacterial adherence to wells. Although visually both *S. sanguis* FW213 and *S. sanguis* JL7 could be seen to adhere evenly to the surface of the polystyrene well when the appropriate concentration of bacteria in carbonate coating buffer (between 10^7 and 10^8 bacteria per well) was used, it seemed desirable to go one step further and quantitate the amount and reproducibility of bacterial adherence. Tritium labeling of a known quantity of *S. sanguis* FW213 or JL7 allowed calculation of the number of counts per minute per streptococcal cell. When 4×10^7 cells were originally added to each polystyrene well, it was found that 2.04×10^7 FW213 and 2.30×10^7 JL7 adhered after nine washes in PBST. Since many investigators are now performing the ELISA in polyvinyl plates, adherence to such surfaces was also measured. Approximately the same number of cells adhered to the polyvinyl plates (1.42×10^7 FW213 and 1.26×10^7 JL7 cells) when an inoculum of equal concentration was used. These results, with the standard deviations obtained, are shown in Table 2.

DISCUSSION

These data show that it is possible to use whole *S. sanguis* cells in the performance of the ELISA technique. The bacteria are seen to coat the wells homogeneously, giving reproducible well-to-well optical densities. The conjugate used in this study was prepared with alkaline phosphatase. Appropriate substrate controls ruled out intrinsic phosphatase activity in the bacteria. If other commercially available conjugates, such as those made with peroxidase or galactosidase, are used in the bactELISA, precautions should be taken to rule out intrinsic enzyme activity. The bactELISA is both simple

TABLE 1. ELISA for *S. sanguis* fimbrial antigens expressed on whole bacteria adsorbed to polystyrene

Anti-FW213	OD ± SD of strain adsorbed to plate[a]:	
	FW213	JL7
Unadsorbed	0.401 ± 0.04	0.311 ± 0.08
Adsorbed with JL7	0.494 ± 0.03	0.051 ± 0.008

[a] Optical density (OD) ± 2 standard deviations was read at 410 nm. The numbers represent averages of six wells. Preimmunization serum showed no absorbance above the blank (wells with no substrate).

and rapid to perform. A similar methodology (effecting bacterial adherence by drying beta-hemolytic streptococci onto the plate in a 60°C oven for 1 h) was utilized in the whole-cell ELISA performed by Cummings et al. (3). These authors, however, made no attempt to determine the final color development with instrumentation. Instead, wells were visually interpreted as positive and negative. Ison et al. (5) adsorbed *N. gonorrhoeae* to polystyrene wells by coating the wells first with poly-*l*-lysine, followed by addition of bacteria, and finally 0.2% gluteraldehyde. In our hands, the use of gluteraldehyde as a bacterial fixative, followed by inactivation with 1 M L-(+)-lysine monohydrochloride, resulted in bacterial adherence, but in addition resulted in a high degree of nonspecific adsorption of rabbit IgG and conjugate. Other investigators (6) have used whole bacterial cells in the ELISA by first coating the well with known antisera, followed by adsorption of the bacterial cell. By adsorbing bacteria directly to the plate, one less coating step is required and less antisera is used. In addition, when testing a large number of clinical isolates for a particular surface structure, it is not necessary to use a group- or species-specific antiserum to which all isolates will adhere.

The method of bacterial adherence chosen here seemed to provide the most consistent and reproducible well coverage. When *S. sanguis* cells were allowed to settle overnight but were not dried, the mutant strain JL7 was seen microscopically to adhere very poorly compared with the parent strain. Rosenberg (9) has reported an observed correlation between adherence of various bacterial species to polystyrene and the hydrophobic differences between the bacterial surfaces of these organisms. The probable loss of two fimbrial types from the surface of JL7 (Fives-Taylor, in press) could account for such a hydrophobic change in this bacterial strain. This loss may also account for the increased variation seen with adsorption of labeled JL7 to both polystyrene and polyvinyl when compared with FW213 (Table 2). Overall, adherence to polyvinyl was less than that to polystyrene for both strains tested. Hydrophobic influences may also be responsible for the decrease seen in whole-cell adherence between the two plates. Our results would seem to indicate that polystyrene may prove superior to polyvinyl in the gram-positive bactELISA.

When *S. sanguis* FW213 was incubated with antiserum before and after adsorption with JL7, a decrease in optical density was not noted. Since adsorption with JL7 should remove reactive antibodies directed against nonfimbrial components of the cell, a decrease in optical density would be expected from the reaction of the adsorbed antiserum and FW213. This decrease was not observed. The continuing high optical density would indicate that a majority of the antibodies produced to *S. sanguis* FW213 are directed against these surface structures involved in adherence. Electron micrographs of strain FW213 revealed that this organism is densely covered with these hairlike fimbriae (Fives-Taylor, in press); hence, their predominance as the recognized antigen is not surprising.

The availability of the bactELISA has considerable potential for workers in the clinical laboratory, as well as for those involved in research. Studies of surface antigens could be simply and rapidly carried out, with the added advantage of the availability of antigen quantitation and the ability to perform a large number of tests at once in one 96-well plate. Typing of somatic, capsular, and flagellar antigens for gram-negative organisms, as well as similar studies on gram-positive bacteria of clinical significance, could be facilitated by the availability of a sensitive enzyme assay which allows for extensive dilution of expensive antisera. In addition, the adsorption of whole bacteria to microtiter well surfaces also lends itself to testing both human and experimental sera for antibodies directed to bacterial antigens, which might be difficult to

TABLE 2. Adherence of *S. sanguis* cells to microtiter plate wells

Strain	No. of *S. sanguis* cells ± 2 SD per well[a]:	
	Polystyrene	Polyvinyl
FW213	$2.04 \times 10^7 \pm 5.6 \times 10^6$	$1.42 \times 10^7 \pm 1.2 \times 10^6$
JL7	$2.30 \times 10^7 \pm 1.2 \times 10^7$	$1.26 \times 10^7 \pm 4.0 \times 10^6$

[a] The numbers represent averages of eight wells.

prepare as purified derivatives. Although different species of bacteria may require various coating conditions, the availability of the bact-ELISA should prove extremely valuable in the study of diverse bacterial cell surface components.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant RO1 05606 from the National Institute of Dental Research.

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