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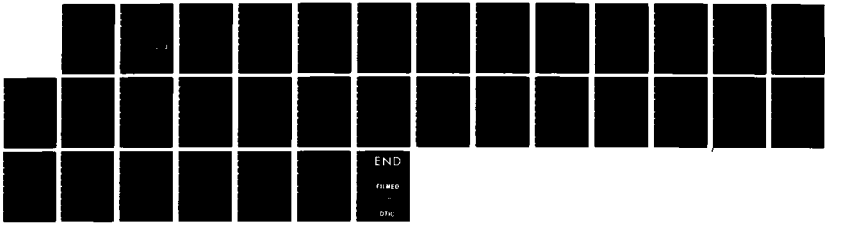

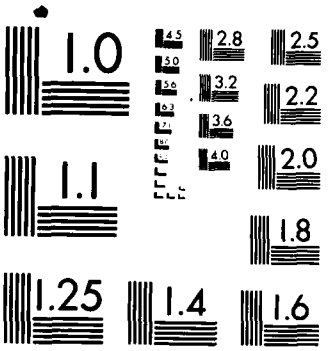

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Eubacterium brachy, a Gram (+) positive anaerobic rod, has been implicated by cultural studies to be associated with the microflora of periodontal diseases. Serum samples from 184 clinically characterized patients were evaluated in a standardized enzyme-linked immunosorbent assay (ELISA) for reactivity to E. brachy antigens. Sera from clinically healthy subjects (HS) served as controls. Sera from rapidly progressive periodontitis (RP) patients demonstrated significantly greater reactivity by ELISA than did HS

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when reactivity with E. brachy antigens was determined ($P < .001$). Juvenile periodontitis (JP) and adult periodontitis (AP) patients did not differ in reactivity by ELISA from HS ($P < .05$). Three to four years following successful periodontal therapy, reactivity was not significantly altered in any patient group ($P > .05$). The possible significance of these findings and the importance of an extracellular antigen of E. brachy in the immunopathology of periodontal diseases are discussed.

(12)

# Systemic Antibody Response of Clinically Characterized Patients With Antigens of Eubacterium brachy Initially and Following Periodontal Therapy*

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# Systemic Antibody Response of Clinically Characterized Patients With Antigens of Eubacterium brachy Initially and Following Periodontal Therapy

## ABSTRACT

Eubacterium brachy, a Gram (+) positive anaerobic rod, has been implicated by cultural studies to be associated with the microflora of periodontal diseases. Serum samples from 184 clinically characterized patients were evaluated in a standardized enzyme-linked immunosorbent assay (ELISA) for reactivity to E. brachy antigens. Sera from clinically healthy subjects (HS) served as controls. Sera from rapidly progressive periodontitis (RP) patients demonstrated significantly greater reactivity by ELISA than did HS when reactivity with E. brachy antigens was determined ($P < .001$). Juvenile periodontitis (JP) and adult periodontitis (AP) patients did not differ in reactivity by ELISA from HS ($P < .05$). Three to four years following successful periodontal therapy, reactivity was not significantly altered in any patient group ($P > .05$). The possible significance of these findings and the importance of an extracellular antigen of E. brachy in the immunopathology of periodontal diseases are discussed.

Numerous review articles provide abundant evidence to implicate microorganisms as the etiologic agent of various periodontal diseases.[1-4] Clinical evidence in support of this concept is abundant. The demonstration that the removal of dental plaque by rigorous oral hygiene[5] or antiseptic agents[6] would prevent or reverse clinical gingivitis in humans is particularly convincing. The description of morphologic changes of dental plaque have been described by Loe et al.[7] beginning with a clinical state of periodontal health through gingivitis and the return to health. Listgarten and Hellden[8] utilized darkfield microscopy to compare the composition of the subgingival flora obtained from healthy and diseased sites from 12 human subjects. Their major conclusion was based on the finding that in normal sites, the ratio of motile to nonmotile microorganisms was 1:49 while from diseased sites this ratio was 1:1. They felt that this information provided a simple and clinically applicable means of detecting disease in humans. Additionally, this method was suggested to be used to monitor the effect of various treatment modalities.

Numerous cultural studies have been reported in which the microflora associated with various periodontal diseases have been described. Slots[9] described the flora isolated from healthy gingival sulci and that associated with gingivitis. Spiegel et al.[10] described the significance of Bacteroides gingivalis in sites demonstrating bone loss and Tanner et al.[11] have implicated Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Bacteroides species and anaerobic vibrios in a variety of periodontal disease sites.

Holdeman et al.[12] recently described the isolation of three new species, Eubacterium timidum, brachy, and nodatum. Only these three species

of Eubacterium, F. nucleatum, and Selenomonas sputigena increased in both incidence and concentration in subgingival samples from sites with severe as compared with chronic periodontitis.[12] In another study evaluating the bacteriology of experimental gingivitis,[13] 166 bacterial species and subspecies were detected among 3,034 randomly selected isolates from 96 samples. Of the numerous microorganisms that appeared to have a positive correlation with gingivitis were several species of Eubacterium including both E. timidum and E. brachy. Other studies evaluating the microbiology of moderate periodontitis[14, 15] consisted of evaluation of 60 samples taken from 38 affected sites in 22 subjects. The species of E. nodatum, timidum, and brachy were among the six most frequently isolated taxa from the subgingival samples of moderate periodontitis while constituting a significantly smaller portion of the flora isolated from supragingival samples of moderate periodontitis or from subgingival samples of healthy sites. Of these three species, E. brachy isolates constituted 2.7% of the flora from moderate periodontitis but was not isolated from supragingival sites or from subgingival sites in healthy subjects.

A similar study was conducted of the flora associated with severe periodontitis.[16] A total of 11 species each exceeded 1% of the subgingival flora and were most closely associated with the diseased sulci. Of these 11 species, five were members of the genus Eubacterium and included the species E. timidum, brachy and nodatum. Recently the microflora associated with diseased sites in 21 individuals with juvenile periodontitis has been described. The three species of Eubacterium were found to be prominent members of the microflora associated with diseased when compared to the microflora of unaffected sites.[17]

The complex nature of the association of microorganisms with the etiology and/or progression of periodontal diseases must be coupled with the role of host defense mechanisms. The purpose of this study was to determine the systemic antibody response to E. brachy antigens with human sera from a large group of clinically characterized patients using a standardized enzyme-linked immunosorbent assay (ELISA). The effect of clinically successful periodontal therapy on antibody titers was also determined for sera samples obtained 3-4 years following completion of therapy.

## MATERIALS AND METHODS

### Patient Selection

Sera from a total of 184 clinically characterized patients were utilized in this portion of the study. A total of 36 patients were classified as healthy subjects (HS) and served as controls. They were selected from periodontally healthy patients seeking restorative care at the University of Maryland Dental School graduate and undergraduate clinics, The Johns Hopkins Hospital and Dental Clinics, and a private practice limited to periodontics. They included age- and sex-matched individuals from the population of dental students, dental hygiene students, faculty, and staff of the University of Maryland Dental School and the Johns Hopkins Hospital. These individuals had periodic (every 3 to 6 months) prophylaxis, gingival[18] and plaque[19] indices of 0.5 or lower, and no historical, clinical or radiographic evidence[20] of periodontal disease.

A total of 44 patients were classified in the adult periodontitis (AP) group. Selection was based on an age ranging from 36 to 45 years and 19 or more teeth. Tooth associated deposits were commensurate with the amount of periodontal destruction, but there was no specific pattern of bone loss.

Forty-nine patients were classified as juvenile periodontitis (JP) patients, a condition previously described as periodontosis. The features of this disease entity have been described as onset around puberty (11 to 13 years of age), increased incidence in females by a ratio of 3:1, and the presence of local factors not always correlated to the degree of periodontal destruction.[21] Additional criteria for selection included the patient's desire to participate in the study, stable residence in the Mid-Atlantic area, 20 or more teeth, and an age range of 12 to 26 years. Juvenile periodontitis patients had a loss of attachment of at least 5 mm on any surface of at least four permanent incisors and first molars.

Fifty-five individuals were classified as rapidly progressive periodontitis (RP) patients. These patients had loss of attachment of at least 5 mm on the surface of at least 14 permanent teeth not limited to incisors and first molars. The criteria utilized for patient selection included: rapid destruction (seen most commonly in teenagers and young adults), extreme inflammation in the active phase of the disease, defects in either neutrophil or monocyte chemotaxis, and periods of exacerbation and quiescence. This disease entity is clinically similar to severe periodontitis.[22]

The clinical and radiographic evaluation of the four patient groups have been previously described.[23] Patients with systemic disease or who

were pregnant were excluded from this study. Also, long-term medical therapy or the taking of antibiotics six months prior to examination excluded patients from the study.

Venous blood was drawn into syringes, allowed to clot at room temperature, and subjected to centrifugation at 500 x g at 4° C. The serum was removed, heat treated at 56° C for 30 minutes, and frozen at -20° C.

Bacterial Cultivation and Antigen Preparations

Cultures of E. brachy (ATCC 33089)* and clinical isolates D79G-22, D82M-1, D4A-24, 1289D and D82L-17 obtained from Virginia Polytechnical Institute; Blacksburg, VA) were grown in a peptone-yeast extract-glucose medium (PYG). Cultures were incubated anaerobically in a BBL† GasPak anaerobic jar system at 37° C. Cultures were streaked for purity on sheep blood agar (BA) plates which were incubated both aerobically and anaerobically. All cultures failed to grow aerobically on BA plates. The cells were harvested by centrifugation at 10,000 x g for 10 min at 4° C, washed 3 X in 0.01 M phosphate buffer containing 0.15 M NaCl (PBS), (pH, 7.2) and suspended in PBS supplemented with 0.01% sodium azide as a 1:10 dilution (V/V) of packed whole cells after centrifugation at 2,000 x g for 10 min at 4° C and was designated as the whole cell preparation (WC).

* American Type Culture Collection; Rockville, MD.

† Baltimore Biological Laboratory; Cockeysville, MD.

In addition, the cell free broth supernatant fluid was filtered using a 0.45 um filter and was subjected to precipitation by mixing 1:8 with absolute methanol at 4° C for 18 h followed by centrifugation at 2,000 x g for 10 min. The precipitate was then suspended in PBS and designated as the broth methanol precipitate (BMP). The BMP (8 ml) was used for isolation of an extracellular antigen (ECA) by gel filtration as previously described.[24] Briefly, on a 2.5 cm x 100 cm column packed with degassed Sephacryl S-200 superfine (Pharmacia Fine Chemicals; Piscataway, NJ) equilibrated with PBS at a flow rate of 1 ml/min, fractions (5 ml) were collected on an Isco Fraction Collector UA-5 monitored with a type 6 optical unit at 280 nm. A P-3 peristaltic pump was utilized to ensure uniformity of flow. Blue dextran ($2 \times 10^6$ Daltons), aldolase (158,000 Daltons) ovalbumin (45,000 Daltons) chymotrysinogen A (25,000 Daltons) and ribonuclease A (13,700 Daltons) were used as standards. Fractions demonstrating absorbance at 280 nm were tested by double diffusion in agar against a human serum previously shown to be reactive with sonicated WC by double diffusion in agar and those fractions demonstrating a precipitation reaction were pooled, precipitated 1:8 in absolute methanol at 4° C, harvested by centrifugation and suspended in PBS at the original concentration. A sample was again precipitated 1:8 in methanol and the precipitate was harvested by centrifugation and lyophilized. Following determination of dry weight, the percent protein (32%) was determined by the Lowry reaction,[25] utilizing bovine serum albumin fraction V* as the standard, and the percent carbohydrate (68%)

* Sigma Scientific Co.; St. Louis, MO.

as the standard, and the percent carbohydrate (68%) was determined by the Anthrone reaction.[26]

Serological Evaluation by ELISA

Previous evaluations by immunoelectrophoresis have demonstrated that the reactivity of human sera with sonicated WC resides in IgG antibody. In addition, lines of identity are shared by sonicated WC and BMP with a reactive human serum.[24]

Six human sera previously shown to be reactive with E. brachy by double diffusion in agar and six sera which did not react with E. brachy were subjected to evaluation by a modification of an enzyme-linked immunosorbent assay (ELISA).[27] Briefly, a 200 ul aliquot of a 1:10 dilution of a previously described WC preparation in 60 mM carbonate buffer (pH, 9.6) was added to each well of a 96-well microtiter plate* and incubated at 37° C for 3 h. Peripheral rows were not utilized. The WC preparation was removed and each well was washed 5 X with 0.01 M PBS containing 0.05% Tween 20. Each well then received 200 ul of a 1% bovine serum albumin*$_1$ suspension in 60 mu carbonate buffer and incubated for 18 h at 4° C. Following washing 5 X with PBS Tween 20, serial twofold dilutions of each serum (100 ul) were added to the appropriate wells and the plates incubated for 30 min at 37° C. The plates were again washed 5 X with PBS Tween 20 and received

* Dynatech Laboratories, Inc.; Alexandria, VA.

*$_1$ Grand Island Biological Co.; Grand Island, NY.

100 ul of a 1:300 dilution of peroxidase conjugated goat anti-human gamma, alpha or mu heavy-chain specific* serum and the plates were incubated again at 37° C for 30 min. After again washing the plates 5 X, 100 ul of the enzyme substrate (1 ml of 1% [wt/v] O-phenylenediamine in absolute methanol combined with 99 ml of distilled water and 0.1 ml of 3% $H_2O_2$) was added. The plates were incubated in the dark for 30 min at room temperature, the reactions were stopped by the addition of 50 ul of 8 N $H_2SO_4$ and the intensity of color resulting was determined colorimetrically at 490 nm with a MR 580 Microelisa Auto Reader.**

Serum samples were absorbed with E. brachy whole cells and subjected to the same ELISA procedures. Briefly 500 ul of each undiluted serum was mixed by vortexing (30 sec) with a 0.2 ml packed volume of the WC and incubated for 1 h at 37° C. The cells were pelleted by centrifugation at 2,000 x g for 10 min and the serum supernatant fluid removed and mixed by vortexing (30 sec) with 0.2 ml of packed WC. Incubation was performed as before and the absorbed serum was again removed following centrifugation. The adsorption step was repeated, as before for a third time prior to evaluation by the ELISA. Reactivity was determined as the reciprocal of the highest dilution giving an OD reading 0.1 greater than microtiter wells receiving the identical treatment as above with the exception of receiving PBS in place of human serum.

* Cappel Laboratories, Inc.; Cochranville, PA.

** Dynatech Laboratories, Inc.

Preliminary attempts to utilize the ECA in an ELISA were not successful due to the failure of the ECA to bind to the microtiter plate. A modification to the conventional procedure was designed by first coating the wells with rabbit anti-ECA serum (1:10, 1:50 and 1:100) in 60 mM carbonate buffer (pH, 9.6). Other modifications attempted included coating the wells with bovine serum albumin fraction V (BSA) (Sigma Scientific Co.) at concentrations of 0.5%, 1%, or 2% in 60 mM carbonate buffer or coating with poly L-lysine (Sigma Scientific Co.) at concentrations of 5 mg/ml, 1 mg/ml, 0.5 mg/ml, or 0.1 mg/ml in 60 mM carbonate buffer. All plates were then incubated for 3 h at 37° C. Following washing 5 X in PBS with 0.05% Tween 20, each well received 200 ul of ECA (400 ug/ml) in the same carbonate buffer and were incubated for 18 h at 4° C. The ELISA was repeated as before utilizing a serum reactive to E. brachy. As shown in Table 1 when the ELISA was performed utilizing the ECA without any means of facilitation of binding, reactivity was minimal. However, when wells were precoated with anti-ECA serum, BSA or poly L-lysine prior to incubation with the ECA, reactivity was greatly facilitated. As a control, wells which had received the anti-ECA serum and all other components of the assay, with the exception of ECA, did not demonstrate appreciable nonspecific binding. However, to utilize this "sandwich" technique it was felt that it would be necessary to utilize this same control for each serum to be tested, which did not appear to be feasible. Based on this fact, and that BSA is normally used as a blocking agent in the ELISA it was decided to utilize BSA (1%) to precoat all microtiter wells prior to utilizing the ECA in ELISA procedures.

All clinically characterized human serum samples were evaluated by the previously described ELISA utilizing the following modifications:

1. All serum samples were diluted 1:20 in PBS/Tween and tested in duplicate.

2. A 1:600 dilution of affinity purified, peroxidase-conjugated goat anti-human gamma, alpha or mu chain serum was utilized.

All assays were run at the same time on the same day in order to standardize incubation temperatures and buffers. The microtiter plates were all from the same lot, and sufficient antiserum, substrate, and sulfuric acid were prepared from the same lots to be used for all assays. The same sera were reacted by ELISA with the ECA. All steps in the procedure were identical with the exception of precoating the microtiter wells with 1% BSA in 60 mM carbonate buffer (pH 9.6) for 3 h at 37° C and incubating the microtiter plates for 18 h at 4° C after the addition of the ECA (400 ug/ml) in the same pH carbonate buffer. Statistical analysis was performed utilizing the _t_ test for unequal N's.[34] Titers were expressed as the highest dilution which resulted in an OD reading 0.1 greater than control wells which had received the identical treatment with the exception that PBS with 0.05% Tween 20 was utilized in place of the human sera.

Serum samples were obtained prior to therapy and additional samples were obtained three-four years following completion of periodontal therapy on selected patients. Of the total patient population, 13 of the 35 healthy subjects, 41 of the 49 juvenile periodontitis subjects, and 38 of the 55 rapidly progressive periodontitis patients provided serum samples three-four years following completion of therapy. None of the adult periodontitis patients provided post-treatment serum samples. All patients

included in this study were clinically classified as well-maintained.

## RESULTS

The reactivity of serum samples with the WC and the ECA of E. brachy is demonstrated in Tables 2 and 3. As can be seen when the ELISA was performed utilizing anti-heavy chain specific serum, the majority of reactivity with the bacterial whole cells of E. brachy resided with IgG with only minimal reactivity detected with IgM and no reactivity with IgA (Table 2). When the ELISA was repeated utilizing the ECA, all detectable reactivity resided with IgG and this reactivity was greatly reduced by prior adsorption of these serum samples 3 X with E. brachy whole cells (Table 3). Prior adsorption of reactive sera 3 X with E. brachy whole cells reduced titers of from 4 to 5 dilutions with the WC preparation (Table 2) and from 3 to 6 dilutions with the ECA (Table 3).

The healthy subjects were comprised of 36 subjects which had been found to be free of any clinical or radiographic signs of periodontal disease (HS). Table 4 presents the data obtained when these sera were reacted by ELISA with E. brachy whole cell preparations and with the ECA. Serum samples were obtained from selected patients three-four years post-initial examination and are represented as post-treatment results. A total of 44 patients had been classified as adult periodontitis patients (AP) based on clinical and radiograph examinations. Serum samples were obtained from these patients prior to the initiation of any therapy. However, no post-treatment sera were available for this group of patients. Table 4 presents the data obtained when these sera were reacted by ELISA with the E. brachy

WC preparation and with the ECA. A total of 49 patients had been classified as juvenile periodontitis patients (JP) based on clinical and radiographic findings. Serum samples were obtained from these patients prior to the initiation of any therapy. Serum samples were also obtained from selected patients 3-4 years following completion of therapy. These selected patients were classified as well-maintained, having responded favorably to the periodontal therapy rendered. Table 4 represents the data obtained when these sera were reacted by ELISA with the E. brachy WC preparation and with the ECA. A total of 55 patients had been classified as rapidly progressive periodontitis patients (RP) based on clinical and radiographic findings. Serum samples were obtained from these patients prior to the initiation of any therapy. Serum samples were also obtained from selected patients 3-4 years following completion of therapy. These selected patients were classified as well-maintained, having responded favorably to the periodontal therapy rendered. Table 4 represents the data obtained when these sera were reacted by ELISA with the E. brachy WC preparation and with the ECA. In all groups tested, the mean OD obtained when sera were reacted with the WC preparation was higher than the mean OD obtained when sera were reacted with the ECA. However, these differences were not statistically significant ($P > .05$).

When the OD readings obtained when sera from periodontal disease groups were reacted with the WC preparation was compared with the healthy subjects, a definite trend was observed. Reactivity of sera obtained from both the AP patients and JP patients were not significantly greater from HS ($P > .05$) (Table 5). However, reactivity of RP patient sera was significantly greater from these same HS sera ($P < .01$).

When the reactivity, based on the OD readings obtained when sera were reacted with the ECA, observed in the various disease groups was compared with the healthy subjects, the same trend was observed. Reactivity of sera obtained from both the AP patients and JP patients was not significantly different from HS ($P > .05$) (Table 6). Reactivity of RP patient sera was significantly different from these same HS sera ($P < .001$).

The effect of clinically successful therapy on the level of detectable antibody to WC and ECA was determined. A total of 38 sera from RP patients harvested prior to therapy and three-four years after therapy were utilized. Statistical analysis revealed no significant differences in detectable antibody level reactive with either WC or ECA following successful therapy (Table 7).

## DISCUSSION

This study was designed to evaluate human systemic antibody reactive with antigens of E. brachy and to determine the effect of clinically successful periodontal therapy on antibody levels. Attempts to correlate human antibody titers reactive to various periodontopathogens has been accomplished with sera obtained from patients characterized by disease state.[23] The concept under investigation is that, if a particular periodontopathogen is readily isolated from the subgingival microflora associated with the diseased site, then antigenic stimulation should result in circulating specific antibody reactive with these particular microorganisms. Based on this concept and guided by the information obtained from cultural studies of the bacterial plaque associated with various disease states, an attempt was made to correlate systemic antibody titers reactive

with the WC and ECA of E. brachy by an ELISA using a conjugated anti-gamma chain serum. When serum samples obtained from clinically characterized patients prior to any periodontal treatment were reacted with the WC of E. brachy, significantly higher titers were observed in RP patients as compared to HS patients ($P < .01$). This was not observed with sera obtained from JP or AP patients ($P > .05$) (Table 5). When this procedure was repeated by reacting human sera with BSA coated wells containing the ECA, the RP patients again demonstrated higher titers of reactive IgG antibody than did HS patients ($P < .001$), but this was not observed for JP or AP patients ($P > .05$) (Table 6). When these pretreatment values obtained with RP patient sera were compared to the values observed in these same patients' sera obtained three-four years following therapy, there was no significant difference in these titers either to the WC or ECA of E. brachy ($P > .05$) (Table 7). When compared to the cultural data of the cultivability of E. brachy from severe periodontitis, these findings tend to support the possible significance of the increased frequency of isolation in cultural studies of the subgingival microflora observed in severe periodontitis.[16] Additionally, the identification of the major antigenic nature of the ECA of E. brachy, as identified in this study, further supports the possible importance of this material especially in light of the previously demonstrated in vitro osteolytic activity of the ECA.[24] The molecular weight of ECA is such (approximately 170,000 Daltons) that one might expect it to penetrate the junctional epithelium as has been demonstrated by other bacterial materials,[28-30] especially in the presence of hyaluronidase,[31] the relative concentrations of which have been shown to increase in gingival fluid with an increasing gingival index.[32]

When compared to the cultural data of the cultivability of E. brachy from severe periodontitis,[16] the increased titers detected support the significance of this microorganism and the reactivity with the purified ECA suggest a role for this substance in the disease process. Our failure to detect increased titers of antibody in AP patients does not correlate with the cultural data of moderate periodontitis.[15] This failure of correlation may be due in part to the severity of the disease in the patient group we evaluated versus the group from which cultural data was obtained. Finally, when these data are added to previous studies, some of which have characterized the humoral response of locally produced,[33] systemic antibody,[23] or salivary immunoglobulin levels[34] reactive with suspected periodontopathogens in various periodontal diseases, our ability to begin to understand the complex host response in the immunopathology of periodontal diseases may be enhanced.

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Table 1
ELISA Utilizing Anti-ECA, Bovine Serum Albumin Fraction V or Poly L-Lysine to Facilitate Binding of ECA

| Pre-coating Treatment | Titer Obtained* |
|---|---|
| No pre-coating | 1:20 |
| Anti-ECA 1:10 | 1:1280 |
| Anti-ECA 1:50 | 1:1280 |
| Anti-ECA 1:100 | 1:640 |
| BSA 2% | 1:1280 |
| BSA 1% | 1:1280 |
| BSA 0.5% | 1:320 |
| Poly L-lysine 5 mg/ml | 1:1280 |
| Poly L-lysine 1 mg/ml | 1:320 |
| Poly L-lysine 0.5 mg/ml | 1:160 |
| Poly L-lysine 0.1 mg/ml | 1:40 |

* Reciprocal of the highest dilution resulting in an OD reading 0.1 greater than microtiter wells receiving the identical treatment with the exception of receiving PBS in place of the reactive human serum.

Table 2
ELISA[a] Titers Obtained With WC of *E. brachy* and Heavy Chain Specific Antiserum

| Reactive Sera | Titer With Anti-gamma | Titer With Anti-mu | Titer With Anti-alpha |
|---|---|---|---|
| 1 | 7 (2)[b] | 2 (0) | 0 |
| 11 | 6 (1) | 1 (0) | 0 |
| 14 | 4 (0) | 0 | 0 |
| 108 | 7 (2) | 1 (0) | 0 |
| 111 | 6 (2) | 2 (0) | 0 |
| 114 | 7 (2) | 2 (0) | 0 |
| Nonreactive Sera | | | |
| 4 | 2 (0) | 1 (0) | 0 |
| 101 | 1 (0) | 0 | 0 |
| 102 | 1 (0) | 0 | 0 |
| 107 | 1 (0) | 0 | 0 |
| 116 | 1 (0) | 0 | 0 |
| 121 | 2 (0) | 0 | 0 |

[a] Highest serum dilution resulting in OD reading greater than 0.1 when compared with control after transforming the results from Geometric Progression to Arithmetic Progressions (1:10 = 1, 1:20 = 2, 1:40 = 3, etc.).

[b] Titer after adsorption of sera 3 X with *E. brachy* WC.

Table 3
ELISA Titers Obtained With the ECA of E. brachy and Heavy Chain Specific Antiserum[a]

| Reactive Sera | Titer With Anti-gamma | Titer With Anti-mu | Titer With Anti-alpha |
|---|---|---|---|
| 1 | 7 (2)[b] | 0 | 0 |
| 11 | 6 (2) | 0 | 0 |
| 14 | 5 (0) | 0 | 0 |
| 108 | 7 (1) | 0 | 0 |
| 111 | 6 (2) | 0 | 0 |
| 114 | 6 (3) | 0 | 0 |
| Nonreactive Sera | | | |
| 4 | 1 (0) | 0 | 0 |
| 101 | 1 (0) | 0 | 0 |
| 102 | 1 (0) | 0 | 0 |
| 107 | 1 (0) | 0 | 0 |
| 116 | 1 (0) | 0 | 0 |
| 121 | 2 (0) | 0 | 0 |

[a] Highest serum dilution resulting in an OD reading greater than 0.1 when compared with control after transforming the results from Geometric Progressions to Arithmetic Progressions (1:10 = 1, 1:20 = 2, 1:40 = 3, etc.).

[b] Titers obtained using the same serum samples which had been adsorbed 3 X with E. brachy whole cells.

Table 4
ELISA Results Representing the OD Obtained When 1:20 Dilutions of Clinically Characterized Sera Were Reacted With the WC and ECA of *E. brachy*

| Patient Population | OD With WC Preparation[a.] | OD With ECA Preparation |
|---|---|---|
| HS Pretreatment N = 36 | 0.353 (0.171) | 0.280 (0.152) |
| HS Post-treatment N = 13 | 0.296 (0.146) | 0.310 (0.229) |
| AP Pretreatment N = 44 | 0.361 (0.199) | 0.304 (0.181) |
| AP Post-treatment N = 0 | b. | b. |
| JP Pretreatment N = 49 | 0.371 (0.149) | 0.315 (0.137) |
| JP Post-treatment N = 41 | 0.351 (0.112) | 0.304 (0.118) |
| AP Pretreatment N = 55 | 0.696 (0.235) | 0.606 (0.210) |
| RP Post-treatment N = 38 | 0.716 (0.256) | 0.607 (0.221) |

a. mean (± standard deviation)

b. serum not available for assay

Table 5
OD Obtained by ELISA by Reaction of Pretreatment Patient Sera With the WC Preparation of E. brachy

| Patient Classification | OD Reading Mean ± SD | T Value | Level of Significance |
|---|---|---|---|
| HS<br>N = 36 | 0.353<br>± 0.171 | | |
| AP<br>N = 44 | 0.361<br>± 0.199 | 0.1897[b] | NS[a] |
| JP<br>N = 49 | 0.371<br>± 0.149 | 0.5092[c] | NS |
| RP<br>N = 55 | 0.696<br>± 0.235 | 7.5362[d] | P < .01 |

[a] NS = not significant, P > .05

[b] Degrees of freedom = 78

[c] Degrees of freedom = 83

[d] Degrees of freedom = 89

Table 6
OD Obtained by ELISA by Reaction of Pretreatment Patient Sera With the ECA of *E. brachy*

| Patient Classification | OD Reading Mean ± SD | T Value | Level of Significance |
|---|---|---|---|
| HS<br>N = 36 | 0.280<br>± 0.152 | | |
| AP<br>N = 44 | 0.304<br>± 0.181 | 0.6528[b] | NS[a] |
| JP<br>N = 49 | 0.314<br>± 0.137 | 1.115[c] | NS |
| RP<br>N = 55 | 0.606<br>± 0.201 | 8.0504[d] | P < .001 |

[a] Not significant, P > .05

[b] Degrees of freedom = 78

[c] Degrees of freedom = 83

[d] Degrees of freedom = 89

Table 7
OD Obtained by ELISA by Reacting Pretreatment and Post-Treatment RP Sera With the WC and ECA Preparations of *E. brachy*

| Patient Classification | *E. brachy* Preparation OD Reading Mean ± SD | T Value | Level of Significance |
|---|---|---|---|
| Pretreatment WC N = 38 | 0.675 ± 0.224 | | |
| Post-Treatment WC N = 38 | 0.716 ± 0.256 | 0.7182[b] | NS[a] |
| Pretreatment ECA N = 38 | 0.589 ± 0.203 | | |
| Post-Treatment ECA N = 38 | 0.607 ± 0.221 | 0.3719[c] | NS |

[a] NS = not significant, P > .05

[b] Degrees of freedom = 74

[c] Degrees of freedom = 74

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