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DEVELOPMENT OF A RAPID QUALITATIVE ASSAY FOR DETERMINING ELEVATED ANTIBODY LEVELS TO PERIODONTOPATHIC ORGANISMS

Brian Luke Mealey

APPROVED:

Supervising Professor

11/29/99

Date

APPROVED:

Sanford A. Miller, Ph.D.

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DEDICATION

This thesis is dedicated to my wife Carla and my two children, Colleen and Patrick, whose love and patient understanding were my inspiration. It is also dedicated to my mother, whose innumerable personal sacrifices I will never forget, and to the memories of my father, which have always given me strength and determination.

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DEVELOPMENT OF A RAPID QUALITATIVE ASSAY FOR DETERMINING ELEVATED ANTIBODY LEVELS TO PERIODONTOPATHIC ORGANISMS

Brian L. Mealey, M.S.

The University of Texas Graduate School of Biomedical Sciences
at San Antonio

Supervising Professor: Jeffrey L. Ebersole

Analysis of serum antibodies to periodontopathic microorganisms has become an integral part of periodontal research. Currently available technology for such analysis is both time consuming and costly. To allow more widespread use in clinical practice, a rapid, qualitative test for determining elevated antibody to periodontitis-associated bacteria was developed. The technique utilizes dot-immunoblotting (DIB) on nitrocellulose with whole formalinized *Actinobacillus actinomycetemcomitans, Bacteroides gingivalis,* and *Bacteroides intermedius*. To enhance its use in a clinical environment, systemic antibody status was determined from whole finger-stick capillary blood. An ELISA was used to compare IgG antibody levels to these microorganisms in peripheral capillary blood and venous serum from 44 subjects. Correlation between serum and capillary levels ranged from r=0.760-0.900 ($p < .0001$). Capillary blood antibody levels averaged 55% of those detected in serum (range: 47-68%). Alkaline phosphatase, horseradish peroxidase (HRP), glucose oxidase, and beta-galactosidase

were tested to determine which enzyme possessed the greatest sensitivity and the most rapid detection of elevated antibody. HRP with 4- chloro-1-naphthol as a colorimetric detector was found to have the best potential in this regard. The optimum concentration of bacterial antigen for distinguishing elevated from normal antibody titers was: *A.a.*, 2.5×10^7; *B.g.*, 1.0×10^7; and *B.i.*, 1.0×10^6, in a 5-10 µl drop of tris-buffered saline. Subsequently, 34 periodontitis patients were analyzed for serum IgG antibodies using a quantitative ELISA. The qualitative DIB assay was performed and results compared in a blind fashion to the ELISA data. Of 12 patients with elevated antibody to *Bg* by ELISA, all 12 were positive in the DIB. Similar results for *Bi* and *Aa* were 13/13 and 10/12, respectively. Analysis of the DIB and ELISA data revealed a sensitivity of 83-100% and a specificity of 84-90% for these three microorganisms. Similar experiments were performed for 10 periodontally healthy subjects. These data were combined with those from the 34 periodontitis patients in order to determine the ability of the ELISA and the DIB assay to delineate the periodontal status of the patients. With these three periodontopathogens, 23/34 (sensitivity = 68%) of the periodontitis patients in this study population were positively identified by the presence of elevated antibody in the DIB assay. Furthermore, 23/27 (positive predictive value = 85%) of the subjects with a positive DIB test had periodontitis. The ELISA and DIB were equal in their ability to delineate periodontal status. A rapid screening assay was developed to determine elevated systemic antibody levels to periodontopathic organisms. The DIB test uses finger-stick capillary blood with results available in less than 2 hours. Using this DIB assay, subjects can be monitored for a change from normal to elevated antibody prior to the clinical detection of disease. Additionally, periodontitis patients can be positively identified by the presence of elevated antibody which is indicative of a specific infection and should lead to more directed treatment regimens. Theses; (KT)

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I. INTRODUCTION

A. Microbiology of Periodontal Diseases

The role of bacteria in the etiology of the periodontal diseases is well established. The composition of the subgingival microflora varies greatly between a state of periodontal health and one of disease. In general, a healthy crevice is populated primarily by nonmotile rods and cocci (Listgarten & Hellden 1978). These are predominantly gram-positive facultative organisms usually of the genera *Streptococcus* and *Actinomyces* (Slots 1977a). A similar microbial composition may be found in previously diseased sites which have undergone clinically successful periodontal therapy (Slots *et al.* 1979). In chronic gingivitis, gram-negative bacteria constitute about 45% and anaerobic organisms about 45% of the total recoverable subgingival flora (Slots *et al.* 1978). Predominant isolates include various species of *Actinomyces*, *Streptococcus*, *Fusobacterium*, and *Bacteroides* as well as *Eikenella corrodens*, and *Capnocytophaga gingivalis* (Slots *et al.* 1978, Savitt & Socransky 1984, White & Mayrand 1981). Acute necrotizing ulcerative gingivitis (ANUG) has been associated with high proportions of *Bacteroides intermedius* and *Treponema* species (Loesche *et al.* 1982).

In advanced adult periodontitis lesions, the cultivable subgingival flora is comprised of approximately 75% gram-negative and 90% anaerobic organisms (Slots 1977b). Common isolates include *Bacteroides gingivalis*, *B.intermedius*, and various species of *Fusobacterium*, *Wolinella*, and non-pigmenting *Bacteroides*.

Perhaps the strongest association between the presence of a particular microorganism and diagnosis of a disease state is that between localized juvenile periodontitis (LJP) and *A.actinomycetemcomitans*. The flora of LJP sites contains mainly gram-negative rods (65%) and anaerobic organisms (Slots 1976). There is a very high frequency of isolation of *A.actinomycetemcomitans* in the subgingival plaque of LJP lesions (generally 90-100%), while the prevalence in periodontally healthy sites is quite low (Slots *et al.* 1980, Mandell & Socransky 1981, Slots *et al.* 1982, Eisenmann *et al.* 1983, Slots & Rosling 1983, Zambon *et al.* 1983a, Mandell 1984, Kornman & Robertson 1985, Asikainen *et al.* 1986, Asikainen *et al.* 1987). Young adults with

generalized advanced periodontal destruction have very high levels of *B.gingivalis* as well as often elevated proportions of *A.actinomycetemcomitans* (Tanner *et al.* 1979). Three serotypes of *A.actinomycetemcomitans* have been detected in the pocket flora. Patients with LJP have serotype *b* twice as often as either serotype *a* or *c* (Zambon *et al.* 1983b). This suggests a high periodontopathic potential of serotype *b*. Indeed, it has been shown that more *A.actinomycetemcomitans* serotype *b* strains produce leukotoxin than do serotype *a* or *c* strains (Zambon *et al.* 1983c).

Much attention has been focused in recent years on the microbial flora associated with advancing, progressive, or "actively destructive" periodontal lesions. While *A.actinomycetemcomitans* appears to be a primary pathogen in rapidly advancing periodontitis in young individuals, *B.gingivalis* has been strongly implicated in rapidly progressing periodontitis in adults (Tanner *et al.* 1979). In recent studies of the flora associated with treated and untreated periodontal lesions which continued to show progressive destruction, *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius*, either alone or in combination, appeared in 99% of progressive lesions but in only 40% of non-progressive sites (Slots 1986, Slots *et al.* 1986). *B.gingivalis* was found in 42% of progressing sites and formed 31% of the cultivable flora, while *B.gingivalis* was seen in only 6% of non-progressive sites and formed only 0.3% of the flora. *A.actinomycetemcomitans* was found in 50% of progressive sites and constituted 0.5% of the flora, while this organism was found in only 5% of non-progressive sites with a median recovery of 0.3%. *B.intermedius* was seen in 58% of progressing lesions with a recovery of 5% of the flora, although this bacteria was seen in 36% of non-progressing sites with a median recovery of only 0.5%. It was determined that the presence of these three organisms above certain threshold levels could distinguish between progressive and non-progressive lesions of adult periodontitis with a sensitivity of 87% and specificity of 84% (Bragd *et al.* 1987). Thus, while acknowledging considerable variability between and within patients, recent literature reveals a significant degree of bacterial specificity in destructive periodontal diseases. Significantly, *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius* appear to be prominently associated with these diseases. This is, of course, not to exclude the

potential importance of other organisms in the pathogenesis of periodontal diseases including *E.corrodens* and various species of *Capnocytophaga*, *Wolinella*, and *Fusobacterium* among others.

B. Host Responses in Periodontal Disease

In the pathogenesis of periodontal disease, there exists a delicate balance between host defense mechanisms and various virulence factors expressed by these organisms. Numerous studies have attempted to elucidate the role of cell-mediated and humoral immune responses in the pathogenesis of periodontal diseases.. Ivanyi & Lehner (1970) and Ivanyi *et al.* (1972) found that plaque-derived organisms stimulated increased lymphocyte transformation in patients with periodontal disease while control organisms did not. Using an experimental gingivitis model (Loe *et al.* 1965), Lehner *et al.* (1974a) evaluated the cell-mediated and humoral responses to plaque derived antigens. They found a bi-phasic response with peak lymphocyte stimulation at 14 and 28 days followed by a decreased response and return to baseline after resumption of oral hygiene. The results of this study and others suggested an initial cell- mediated response of relatively short duration.

Evidence suggests that gingivitis is primarily associated with T-lymphocytes while periodontitis is associated with activation of B-lymphocytes (Mackler *et al.* 1977, 1978a, 1978b, Seymour & Greenspan 1979, Daly *et al.* 1983). Seymour *et al.* (1979a, 1979b) proposed that the shift from a T-cell to a B-cell lesion might be used as an indicator of progression from gingivitis to destructive periodontitis. This was an important early attempt to use immunologic markers as indicators of periodontal disease progression. However, Page & Schroeder (1981) determined that there was no correlation between initiation of destructive periodontitis and conversion from a T-cell to a B-cell lesion. Indeed, the lesion of "established" gingivitis, which does not exhibit attachment loss or bone destruction, is predominated by B-lymphocytes and plasma cells rather than T-lymphocytes (Page & Schroeder 1976).

It is incorrect to consider a particular stage of disease as either a "T-cell" or "B-cell" lesion. In fact, there is extensive interplay between the T cell and B-cell arms of the immune response to plaque-derived antigens (Lehner 1982). Taubman *et*

al.(1984) proposed that T-cells have a regulatory function in the pathogenesis of periodontal destruction via modulation of the B-cell response. Rats lacking in T-lymphocytes demonstrated increased bone loss after infection with *A.actinomycetemcomitans*. When T-lymphocytes were returned to these animals, increased B-lymphocyte proliferation and IgG production with a concomitant decrease in bone loss was seen. Miller *et al.* (1978) also demonstrated that T-lymphocytes were needed for maximal stimulation of human B-lymphocytes. Carpenter *et al.* (1984) showed that IgG and IgM production decreased significantly in vivo when T-cells were depleted from the lymphocyte population. As increasing numbers of T-cells were returned to cultures, the level of IgG and IgM production likewise increased.

The cell-mediated response to oral microorganisms may vary considerably between individuals. Stashenko *et al.* (1983) found that overall, T-cells from healthy and periodontally diseased patients proliferated equally well in response to a number of oral bacteria. However, their subjects individually showed differing responses. Low responders demonstrated increased signs of periodontal inflammation relative to high responders, indicating that patients may respond differently to the same antigenic challenge. Stashenko *et al.* (1985) also found low absolute numbers of lymphocytes and T-cells with decreased T-helper/T-suppressor ratios in low responders relative to medium and high responders. Since patients with low T-helper/T-suppressor ratios had greater tissue inflammation, the authors conclude that the T-helper response may be important in limiting bacterial-induced inflammation.

Controversy exists as to the relative importance of polyclonal versus monoclonal (specific) B-lymphocyte responses in periodontal disease. Bick *et al.* (1981) demonstrated polyclonal stimulation of human peripheral blood lymphocytes in response to plaque-derived antigens/mitogens. Donaldson *et al.* (1982) used periodontitis associated bacteria to test the polyclonal response of peripheral blood lymphocytes from patients exhibiting periodontal health, juvenile periodontitis, moderate adult periodontitis, and severe adult periodontitis. They found a similar frequency and intensity of response in all groups. Suzuki *et al.* (1984a) confirmed the

similarity in polyclonal response of peripheral blood lymphocytes from periodontally healthy and diseased subjects to known mitogens and periodontal pathogens. Thus, polyclonal lymphocyte responses to plaque-derived antigens/mitogens may not be indicative of the relative state of periodontal health.

C. Alterations in Serum Antibody Response in Different Disease Categories

One of the first reports to relate the presence of gram-negative anaerobic organisms in the oral cavity to the presence of elevated serum antibody specific to these organisms was that of Evans *et al.* (1966). They reported elevated serum antibody levels to *Fusobacterium polymorphum* in patients with severe adult periodontitis compared to normal adult sera.

The possibility that patients with LJP may have an altered immune response was first suggested in a report by Lehner *et al.* (1974b) in which total serum IgG, IgM, and IgA concentrations were significantly elevated in LJP and post-LJP patients compared to the levels in normal subjects. This elevation in total serum immunoglobulin concentration has also been seen in patients with the generalized form of juvenile periodontitis (Kaslick *et al.* 1980), although another report showed no elevation in IgM or IgA in these patients, with some elevation of total IgG (Ranney *et al.* 1981). Murray & Genco (1980) found mean total serum IgG levels in LJP patients 25 times greater than mean titers of healthy patients. A number of studies support the finding that IgG is also the major immunoglobulin produced locally in LJP (Van Swol *et al.* 1980, Waldrop *et al.* 1981, Gebhard *et al.* 1982).

Numerous studies have demonstrated a markedly increased prevalence and level of systemic antibody specific for *A.actinomycetemcomitans* in LJP patients compared to all other forms of periodontal disease or health (Genco & Slots 1984). Ebersole *et al.* (1980a) found elevated antibody levels to *A.actinomycetemcomitans* strain Y4 in 74% of patients with LJP compared to 27% of those with the generalized form of juvenile periodontitis and 16% of those with adult periodontitis. Conversely, patients who were edentulous, periodontally healthy, or who had acute necrotizing ulcerative gingivitis (ANUG) demonstrated minimal antibody activity to this organism. Genco *et al.* (1980a,b) found 56-70% of LJP patients had antibody to

A.actinomycetemcomitans compared to only 8-20% of patients diagnosed as healthy, adult periodontitis, generalized juvenile periodontitis, ANUG, post-LJP, or edentulous. Altman *et al.* (1982) found elevated serum antibody to this organism in 70% of LJP patients compared to 19% and 29% of those with adult periodontitis and rapidly progressive periodontitis respectively. Ebersole *et al.* (1982a) and Taubman *et al.* (1982) demonstrated not only an increased prevalence of serum antibody to *A.actinomycetemcomitans*, but also a significantly increased level of antibody in LJP patients compared to all other forms of disease or health. In fact, Ebersole *et al.* (1982a) concluded that the serum antibody response to *A.actinomycetemcomitans* could be used to differentiate LJP patients from all other groups.

Certain strains of *A.actinomycetemcomitans* are known to produce a leukotoxin which destroys human polymorphonuclear leukocytes and monocytes (Tsai *et al.* 1979). Listgarten *et al.* (1981) found elevated serum antibody to three leukotoxin-producing strains of *A.actinomycetemcomitans* (Y4, ATCC 29522, ATCC 29524) in patients with LJP relative to healthy and adult periodontitis subjects. Elevated antibody specific for the non-leukotoxin-producing strain (ATCC 29523) was not detected in any of the groups. Baehni *et al.* (1979) evaluated the effects of sera from patients with juvenile and adult periodontitis, ANUG, and periodontal health on the leukotoxic activity of *A.actinomycetemcomitans* strain Y4. They found that 91% of juvenile periodontitis sera neutralized the leukotoxic activity compared to 24% of normal, 39% of adult periodontitis, and 38% of ANUG sera. Genco *et al.* (1980b) found that 89% of LJP patients possessed antibody which inhibits the leukotoxin compared to 20-30% of healthy or adult periodontitis subjects. This leukotoxin-neutralizing effect of serum from LJP patients was recently confirmed in a study by Kalmar *et al.* (1987).

The presence of serum antibodies to *A.actinomycetemcomitans* in LJP patients has been correlated to the localization and severity of this disease process. Ranney *et al.* (1982) found antibody specific for this organism in 77% of LJP and 40% of generalized juvenile periodontitis subjects compared to only 2% of healthy controls. When the percent of periodontally involved teeth was correlated with the presence of serum antibody, they found that the percent of involved teeth was significantly lower

for those subjects with detectible antibody to the organism than for those without antibody. That is, the presence of antibody was inversely related to the severity of periodontal destruction. This suggests a protective role for antibody to *A.actinomycetemcomitans* in the pathogenesis of juvenile periodontitis. Tsai *et al.* (1981) demonstrated inhibition of *A.actinomycetemcomitans* leukotoxic activity from 90% of LJP sera. Conversely, sera from healthy and adult periodontitis subjects lacked the ability to inhibit leukotoxic activity. The authors suggest that production of antibody specific for *A.actinomycetemcomitans* and its leukotoxin early in the disease process may cause localization and even remission of the disease. If the immune response is delayed or inadequate, the organism and its leukotoxin continue to cause periodontal destruction, leading to a more generalized pattern of disease.

Rapidly progressive periodontitis is a form of periodontal disease characterized by generalized, severe, rapid bone loss in patients between puberty and 35 years of age (Page *et al.* 1983). Terms which have been used to describe similar clinical conditions include advanced destructive periodontitis (Ebersole *et al.* 1982b), severe periodontitis (Ranney *et al.* 1981), and generalized juvenile periodontitis (Van Dyke *et al.* 1982). Ranney *et al.* (1981) evaluated total serum IgG, IgM, and IgA titers in young adults with "severe periodontitis". Serum IgM and IgA levels did not differ from healthy patients, although IgG levels were higher for severe periodontitis patients. However, 8/26 severe periodontitis subjects had abnormally high IgG titers compared to only 1/24 healthy patients. As mentioned previously, Ranney *et al.* (1982) found a prevalence of antibody specific for *A.actinomycetemcomitans* in generalized juvenile periodontitis subjects that was intermediate between that for LJP and healthy subjects. Ebersole *et al.* (1980b) evaluated the IgG and IgM antibody activity specific for *B.gingivalis* in patients with various forms of periodontal disease. They found the highest mean levels of activity to *B.gingivalis* occurred in the patients with generalized juvenile periodontitis. Similar elevated activity was seen in adult periodontitis patients while healthy patients and those with LJP and ANUG had minimal activity. In addition, the prevalence of serum antibody to *B.gingivalis* and *B.intermedius* is increased in patients with rapidly progressive periodontitis compared to healthy and LJP subjects (Ebersole

et al. 1986). This increased prevalence and level of serum antibody to *B.gingivalis* has been confirmed by a number of authors (Altman *et al.* 1982, Suzuki *et al.* 1984b, Taubman *et al.* 1982). Mouton *et al.* (1981) found that patients with generalized juvenile periodontitis had mean serum IgG levels specific for *B.gingivalis* eight times higher than the normal population. Within this group of patients, considerable variability in antibody response was seen, with some patients having a strong response while others had minimal response. In addition to *B.gingivalis* and *B.intermedius*, elevated responses to *Fusobacterium nucleatum* have been seen in patients with rapidly progressive periodontitis (Suzuki *et al.* 1984b, Tew *et al.* 1985). Also, extremely high serum antibody titers to one or more serotypes of *A.actinomycetemcomitans* have been noted in patients with rapidly progressive disease (Genco *et al.* 1985).

The serum antibody response in adult periodontitis is more difficult to characterize than that of juvenile or rapidly progressive periodontitis due to more significant patient variation. Altman *et al.* (1982) found elevated antibody to *B.gingivalis* in 71% of adult periodontitis patients, while elevated antibody to *A.actinomycetemcomitans* was present in only 19%. This compares favorably to the 68% prevalence of increased antibody to *B.gingivalis* in adult periodontitis shown by Ebersole *et al.* (1980b). Most studies confirm the relatively low prevalence of serum antibody to *A.actinomycetemcomitans* in patients with adult periodontitis (Ebersole *et al.* 1980a, Genco *et al.* 1980a, Genco *et al.* 1985). Likewise, a number of studies have affirmed an increased prevalence and level of serum antibody specific for *B.gingivalis* in these patients (Ebersole *et al.* 1986, Taubman *et al.* 1982, Tolo & Brandtzaeg 1982). Mouton *et al.* (1981) found over 50% of adult periodontitis patients had significantly elevated serum antibodies to *B.gingivalis;* furthermore, the level of antibody specific for *B.gingivalis* was five times greater in adult periodontitis than in healthy subjects. Naito *et al.* (1984) confirmed the higher prevalence and level of serum IgG antibody to *B.gingivalis* in adult periodontitis. Importantly, they also found a significant correlation between the severity of periodontal destruction and the degree of elevation of antibody specific to this organism. In addition to *B.gingivalis*, several authors have noted an elevation of the serum antibody response to *F.nucleatum* in patients with

adult periodontitis (Naito *et al.* 1984, Suzuki *et al.* 1984b).

The microbiota associated with ANUG is characterized by a dramatic increase in the proportion and numbers of spirochetes (Listgarten 1965). As previously discussed, Loesche *et al.* (1982) cultured plaque samples from ANUG patients and found a predominance of *B.intermedius* and *Treponema* species. Chung *et al.* (1983) demonstrated a two-fold elevation of serum antibody titers to *B.intermedius* and a three- to eight-fold increase in antibody to intermediate-sized spirochetes in patients with ANUG compared to healthy subjects or patients with chronic gingivitis.

Several authors have reported that certain patients with periodontal disease respond poorly to conventional forms of therapy (Hirschfeld & Wasserman 1978, McFall 1982, Goldman *et al.* 1986). In an attempt to determine possible differences between these so-called "refractory" patients and those who respond well to therapy, Haffajee *et al.* (1988a) studied the clinical, microbiological and immunological features of 27 patients, including 13 who were refractory to treatment. Four of the 13 refractory patients had elevated serum antibody levels to *F.nucleatum* compared to only 1/14 non-refractory subjects. In addition, 5/13 refractory patients exhibited an elevated antibody response to *B.intermedius* while none of the non-refractory patients had an elevated response to this organism.

Research performed in the early 1980s has altered the concept of periodontal destruction as an ongoing, continuous process. While not proven beyond doubt, it is now believed that periodontal destruction occurs in bursts of unknown duration, with intervals of quiescence between these periods of active disease (Goodson *et al.* 1982, Haffajee *et al.* 1983, Socransky *et al.* 1984). Haffajee *et al.* (1988b) recently studied the clinical, microbiological and immunological features of 33 subjects with evidence of active disease. The most frequently detected subgingival bacteria were *F.nucleatum* and *Streptococcus intermedius*. There was considerable variation in organisms recovered from both active and inactive sites within and between patients. Serum antibody responses to the 18 subgingival organisms tested also varied among patients. More than 81% of subjects had an elevated antibody response to at least one organism. Several individuals had elevated responses to two or more organisms, while

one subject showed an elevated response to 5 species. The severity of attachment loss could not be related to any particular antibody response pattern. Interestingly, half of the subjects with active periodontal destruction demonstrated elevated serum antibody responses to one or more serotypes of *A.actinomycetemcomitans*. In this study, the organisms recovered from subgingival sites in high numbers did not necessarily correlate with presence of an elevated antibody response to that particular organism. This is not surprising in light of the possibility that the predominant organism in a particular site may have little or no pathogenic potential. The authors conclude that "active" periodontal disease is not a single entity with specific clinical, microbiological, and immunological parameters. Rather, multiple distinctive periodontal diseases are represented in this population of subjects, all of which may undergo periods of active tissue destruction.

Ebersole *et al.* (1987a) evaluated a group of 13 LJP, rapidly progressive, and adult periodontitis patients over a 4.5 year period. During this time period 12/13 subjects experienced at least one episode of active periodontal destruction, with 5 patients having multiple episodes (2-6). Serum antibody studies showed an elevated response to at least one of the 15 subgingival organisms tested in 11 of 13 subjects, with 7/13 having an elevated response to *A.actinomycetemcomitans*, 5/13 to *B.gingivalis*, 4/13 to *E.corrodens*, and 3/13 to *B.intermedius*. These results confirm that most patients with periodontal diseases exhibit elevated serum antibody responses to one or more proposed periodontal pathogens.

Ebersole *et al.* (1984a) evaluated the relationship between elevated serum antibody levels and colonization by the homologous organism. The presence of an organism was related to the presence or absence of an elevated antibody response to that organism in 34 patients with LJP, adult periodontitis, or advanced destructive periodontitis. All patients had elevated serum antibody to one or more of 19 subgingival organisms tested. There was an 81% agreement between elevated antibody to a species and detection of that organism. These data strongly suggest that an elevated systemic antibody response to an organism reflects infection with the species and subsequent host recognition of a specific bacterial infection. The authors

conclude that monitoring of the systemic antibody responses may aid in recognition of potential pathogens in periodontal disease.

Genco *et al.* (1985) also studied the specificity of the systemic antibody response relative to the presence or absence of the same organism. They evaluated the immune response to *A.actinomycetemcomitans* in 6 LJP patients to determine if the antibody specificity reflected the serotype of the organism found in diseased subgingival sites. Three of the patients were only infected with serotype *a* strains of *A.actinomycetemcomitans* while the other three patients were infected with serotype *b* strains. They determined that sera from subjects infected with serotype *a* strains reacted strongly to the autologous serotype *a* strains while those infected with serotype *b* had high serum antibody titers to autologous serotype *b* strains. This indicates that the systemic antibody response to *A.actinomycetemcomitans* is highly specific and is directed mainly to the serotype antigens on the species strains infecting each subject. These data provide additional evidence that serum antibody production results from infection of the periodontium with a particular organism.

Ebersole (1987a) performed a similar study in 13 subjects comparing the presence of elevated systemic antibody and the homologous microorganism in disease-active versus disease-inactive intraoral sites. They found that 80% of the subgingival samples from disease-active sites contained the same organism to which the subject demonstrated an elevated systemic antibody response. In contrast, only 20% of disease-inactive sites showed a similar relationship. The authors conclude that the presence of elevated serum antibodies reflect the host immune response to an infection associated with an episode of disease activity.

A comparable study was performed to evaluate the microflora associated with disease-active and disease-inactive sites and to compare these data with the serum antibody response to a battery of 18 oral microorganisms (Ebersole *et al.* 1987b). Thirty-four patients with various types of periodontal disease were monitored longitudinally to assess disease activity. Subgingival plaque samples were cultured from active and inactive sites. While most of the species of organisms were cultured with similar frequency from active and inactive sites, some species were isolated

primarily from active sites. In particular, *A.actinomycetemcomitans, B.gingivalis, Wolinella recta, E.corrodens,* and to a lesser extent *B.intermedius* were predominantly associated with disease-active sites. All of the subjects had elevated antibody titers to at least one of the 18 organisms tested. The elevated serum antibody response was found to agree with intraoral colonization by the same organism approximately 85% of the time. Furthermore, when an individual exhibited elevated antibody titers to a particular organism, that organism was found in 55% of disease-active sites, but in only 18% of disease-inactive sites. These data indicate that not only does an elevated systemic antibody response indicate oral colonization by particular organisms, but these bacteria are most frequently associated with subgingival plaque in sites of active disease.

D. Serum Antibody Changes During Periodontal Therapy

In most of the previously mentioned studies, the systemic immune responses were examined in an effort to characterize or categorize periodontal disease patterns. In contrast, a number of recent studies have evaluated the serum antibody response following periodontal therapy. Tolo *et al.* (1982) obtained serum samples from 12 patients with adult periodontitis before and one year after periodontal surgery with scaling and root planing. Before therapy, all 12 diseased patients had elevated IgG antibody specific for *B.gingivalis*. One year after treatment, 7 of 12 subjects had a significant decrease of 25% or more in antibody titer to this organism, while the antibody level in the other 5 patients remained the same. In no subjects did the serum antibody level to *B.gingivalis* increase after therapy. Except for *B.gingivalis*, the majority of the test species showed specific systemic antibody activities within the normal range both before and after treatment. It is important to note that these subjects received no maintenance therapy after the first three months following surgery.

Sandholm & Tolo (1986) found similar results in patients with juvenile periodontitis. Sixteen to 32 months after treatment, systemic antibody titers to *A.actinomycetemcomitans, B.gingivalis, C.ochracea,* and *Eubacterium saburreum* either decreased or remained similar to baseline levels. In this study and in Tolo *et al.*

(1982), antibody levels were not analyzed during the first year post-treatment; thus, no conclusions can be drawn regarding the dynamics of the antibody response immediately after therapy.

Ebersole *et al.* (1985) evaluated the effects of scaling and root planing on serum IgG antibodies to a battery of oral microorganisms in 31 subjects classified as either LJP, adult periodontitis, advanced destructive periodontitis, or periodontally healthy. The subjects were monitored clinically and immunologically on a bi-monthly basis for up to 27 months. Nineteen patients exhibited disease activity during the monitoring period and subsequently underwent scaling and root planing. The other 12 subjects received no scaling. Before scaling, 96% of the diseased subjects had an elevated serum antibody response to one or more of the organisms tested. Significant increases in antibody levels were noted in 16 of 19 patients shortly after scaling compared to only 2 of 12 nonscaled subjects. The authors suggest that the treatment rendered may have actively immunized the patients with bacteria colonizing the gingival crevice. In 42% of the cases where an elevation in antibody level to an organism was detected before treatment, a significant increase was seen after therapy. By comparison, increases in antibody level post-treatment were demonstrated in only 8% of the cases where pre-treatment levels were normal. The kinetics of the antibody response differed in treated versus untreated subjects. In untreated patients, the antibody levels remained relatively constant over the monitoring period. However, in patients who received scaling and root planing, significant increases in serum antibody titers were seen, with the response level peaking about 2 to 4 months after therapy. Furthermore, in subjects who had multiple episodes of scaling, this increase in antibody level to some organisms was seen after each treatment. In general, antibody levels returned to normal by 8 to 12 months. The post-treatment increase in antibody level was most frequently directed toward *B.gingivalis*, *B.intermedius*, *E.corrodens*, and *A.actinomycetemcomitans*, a finding consistent with the high frequency of colonization by these organisms in periodontal lesions. In 18 of 19 circumstances, the homologous species was present in the subgingival plaque when elevated antibody titers were present after treatment. The authors conclude that increased antibody levels after

treatment were indicative of a host response to an organism present in the subgingival flora before scaling. It was noted, however, that organisms could also be detected which did not elicit an increased antibody response after therapy.

Ebersole *et al.* (1987a) later confirmed these results by demonstrating elevation of systemic antibody levels to one or more organisms in 73% of subjects following scaling and root planing, with a peak titer 3 to 4 months post-treatment. Mouton *et al.* (1987) performed similar research specifically directed at *B.gingivalis* in 11 adult periodontitis and 9 healthy patients. Of the 11 diseased subjects, 5 had normal pre-treatment levels of serum IgG antibody to this organism while 6 had significantly elevated titers. Following scaling and root planing, patients in the initially low-reactive group maintained low levels of antibody to *B.gingivalis*. In the initially high-reactive group, antibody titers demonstrated a progressive decline to 55% of the pre-treatment level 5-7 months after treatment and 41% at 1 year post-treatment. However, this decreased level of antibody to *B.gingivalis* after 1 year was still 6 times higher than that of periodontally healthy patients. In contrast to Ebersole *et al.* (1985, 1987a) and in agreement with Tolo *et al.* (1982), these authors concluded that periodontal therapy does not precipitate increased levels of serum antibody to *B.gingivalis*. As acknowledged by Ebersole *et al.* (1985), not all species will elicit an elevated antibody response after therapy. Mouton *et al.* (1987) suggest that *B.gingivalis* may be such an organism.

Vincent *et al.* (1987) evaluated the serum antibody responses following periodontal therapy in subjects with juvenile and rapidly progressive periodontitis and compared them to periodontally healthy patients. Both juvenile and rapidly progressive periodontitis patients demonstrated significantly elevated pre-treatment antibody levels to *B.gingivalis*, *A.actinomycetemcomitans*, and *F.nucleatum*. Immediately following treatment (within one month after surgery), juvenile periodontitis subjects showed a significant increase in serum antibody titers to these organisms. Three to fours years after therapy, antibody titers had declined to below pre-treatment levels; however, the titers remained significantly greater than that seen in healthy subjects. Unlike those with juvenile periodontitis, rapidly progressive

periodontitis subjects demonstrated no significant difference between pre-treatment and immediate post-treatment levels. Three to fours years after therapy, these subjects also displayed a significant decrease in antibody titer specific for *B.gingivalis* and *F.nucleatum* to a level below pre-treatment but significantly greater than healthy individuals. Antibody levels to *A.actinomycetemcomitans* were not significantly different from pre-treatment to 3-4 years post-treatment in rapidly progressive periodontitis subjects. Thus, post-treatment systemic antibody responses may be different in these two periodontal disease catagories.

Aukhil *et al.* (1988) evaluated serum antibody titers to potential periodontal pathogens at various intervals in treatment. They noted considerable individual variability in the magnitude of response to various organisms at different time points. In general, there was a significant decrease in antibody titers to *B.gingivalis*, *B.intermedius*, and *S.sanguis* following scaling and root planing. Furthermore, the cumulative changes resulted in a significant decrease in titer to almost all organisms tested by the end of the maintenance phase, two years after scaling and root planing. Interestingly, for a small percentage of subjects (26%), scaling and root planing was followed by a significant increase in antibody titers to one or more organisms. It is important to note that results of studies regarding the effect of periodontal therapy on systemic antibody responses are difficult to compare directly due to differences in methodology and patient populations. It is obvious however, that periodontal therapy does result in significant alterations in the host systemic immune response.

E. Use of Antibody Determinations

For many infectious diseases, detection of serum antibodies provides significant diagnostic potential. The clinical and radiographic features of the periodontal diseases are often sufficient to make a diagnosis based on commonly used criteria. However, a number of aids are now available to assist in making difficult diagnostic decisions (Kornman 1987). The detection of serum antibody to various organisms may provide a needed clue if diagnostic problems arise (Taubman *et al.* 1982).

As mentioned previously, elevations in serum antibody titers to particular organisms are associated with several of the periodontal diseases. Ebersole *et al.*

(1982b) attempted to classify the antibody response patterns in patients with various forms of periodontal disease. They found at least seven characteristic responses: I) elevated antibody to *A.actinomycetemcomitans* only, II) elevated antibody to *A.actinomycetemcomitans* and very low titers (lower than normal population) to *Capnocytophaga sputigena*, III) high antibody titers to both *A.actinomycetemcomitans* and *B.gingivalis*, IV) high titers to *B.gingivalis* only, V) elevated antibody to a single gram-negative species other than those mentioned, VI) elevated titers to three or more gram-negative species, and VII) no elevation in antibody titer to any of the organisms tested. Nearly 90% of LJP patients fell into catagories I, II, or III; that is, they had elevated titers to *A.actinomycetemcomitans*, either alone or in combination with other distinctive responses. Approximately 58% of subjects with adult periodontitis had elevated antibody to *B.gingivalis* (category III or IV), while a similar response pattern was seen in 50% of patients with advanced destructive periodontitis. Greater than 85% of periodontally healthy subjects were in category VII; i.e., no elevation in antibody titer to any of the organisms. Only 3% of LJP, 2% of advanced destructive periodontitis, and 17% of adult periodontitis subjects fell into this category. It is obvious that there are major variations in systemic antibody responses among periodontal disease catagories. The authors conclude that the information obtained by analysis of serum antibody responses could be useful in defining and differentiating various types of periodontal disease based on a combination of clinical and laboratory data.

In a subsequent study (Ebersole *et al.* 1984a), an attempt was made to predict, based on clinical disease parameters and presence or absence of disease activity, into which antibody categories subjects would fall. In the 35 patients evaluated, there was a 79% agreement between predicted and actual antibody response patterns, suggesting an association between the clinical parameters and specific antibody responses.

Cenco *et al.* (1985) assessed the value of serum IgG antibody determinations specific for *A.actinomycetemcomitans* in the diagnosis of LJP. The sensitivity of their antibody tests, defined as the number of LJP patients exhibiting a positive test

compared to the total number of LJP patients tested, was 71%. The specificity, defined as the number of non-diseased subjects with a negative test compared to the total number of non-diseased subjects, was 89%. Thus, 71% of patients diagnosed with LJP had an elevated antibody response to *A.actinomycetemcomitans*. Likewise, only 11% of periodontally normal individuals showed elevation in antibody response to this organism, a finding consistent with the 10% level of *A.actinomycetemcomitans* infection reported in this group of normal subjects (Zambon *et al.* 1983a). The predictive value of a positive test, defined as the number of positive tests in LJP subjects divided by the total number of positive tests, was 86% while the predictive value of a negative test (number of negative tests in LJP subjects divided by the total number of negative tests) was 75%. The authors conclude that when used alone, the determination of serum IgG antibody specific for *A.actinomycetemcomitans* is quite accurate in diagnosing LJP. They further suggest that evaluating serum antibody levels to this organism may be helpful in the diagnosis of other forms of *A.actinomycetemcomitans*-associated periodontal diseases.

It is well established that LJP tends to occur within certain families (Zambon *et al.* 1983a). Zambon *et al.* (1983a) proposed that intrafamilial transmission of *A.actinomycetemcomitans* may partly explain the familial tendency of this disease. Determination of serum antibody response to this microorganism has been suggested as a means of identifying family members at risk for developing the disease. Genco *et al.* (1985) examined serum IgG, IgM, and IgA antibody titers to *A.actinomycetemcomitans* in diseased and non-diseased individuals in the same families. The LJP- affected subjects had significantly greater IgG and IgA antibody titers than did non-diseased siblings. Furthermore, the IgG, IgA, and IgM titers in LJP-affected siblings were significantly elevated compared to periodontally healthy, unrelated subjects. Interestingly, periodontally normal siblings of LJP patients had significantly elevated IgM antibody titers to *A.actinomycetemcomitans* compared to unrelated normal subjects. In fact, the IgM titers in healthy siblings were almost equal to the IgM titers in their LJP-affected relatives. Thus, in LJP families, non-diseased siblings can be distinguished from their diseased relatives based on the lack of

elevation of IgG and IgA antibodies to *A.actinomycetemcomitans* in the healthy individuals. However, the increased IgM titers in non-diseased siblings suggests that they have indeed been exposed to the organism. The authors propose that the elevated IgM response may precede clinical signs of LJP in these subjects. Thus, determination of serum antibody responses may help predict future periodontal destruction. This may lead to frequent periodontal evaluation of these patients and possibly early intervention in the disease process, thus preventing the rapid destruction of periodontal support characteristic of LJP (Novak *et al.* 1988).

Serum antibody studies have been used to predict the response to periodontal therapy; that is, to assess the prognosis and alter treatment accordingly. Ebersole *et al.* (1984a) compared the hazard rate (ratio of the number of sites experiencing active disease during the monitoring period to the total number of sites at risk) to the antibody response category (I-VII) both before and after treatment. When active disease was detected, surgical therapy with adjunctive systemic tetracycline was provided. Patients in categories V and VI (elevated antibody to one gram-negative species and to three or more species respectively) demonstrated the highest hazard rates before treatment. After treatment, the hazard rates decreased in all patients; however, patients who had elevated antibody titers to three or more gram-negative organisms (category VI) responded poorly to therapy and continued to have post-treatment hazard rates significantly higher than all other subjects. The authors suggest that categorizing patients into antibody response patterns may provide important prognostic information and allow design of more effective treatment modalities for these individuals.

Determination of specific serum antibody titers may be useful in guiding periodontal therapy. It has been shown that in 85% of the instances when elevated serum antibody to a species is demonstrated, the same organism will be found intraorally; furthermore, these bacteria are most frequently associated with subgingival plaque from sites exhibiting disease activity (Ebersole *et al.* 1987b). Thus, finding elevated antibody titers to an organism or organisms may lead to treatment aimed at eradication of that species from the subgingival environment either by mechanical

treatment, adjunctive antibiotic therapy, or both.

Aukhil *et al.* (1988) demonstrated a generalized decrease in serum antibody titers to subgingival bacteria one to two years after periodontal therapy. The authors suggested that analysis of specific serum antibody titers might be useful in monitoring of patients in the maintenance phase of therapy. Due to the sensitivity of the anamnestic response, maintenance of an immune response to subgingival organisms should require minimal levels of antigenic stimulation in a patient previously sensitized to that antigen. Thus, in a patient who has been sensitized to bacteria associated with periodontal disease, it is reasonable to expect a rapid rise in serum antibody titer if that organism again appears in significant numbers in the subgingival flora. Of course, the amount of bacteria needed to stimulate such a rise in antibody titer depends on the relative immunogenicity of the organism. An elevation in antibody level might then suggest that additional therapy is indicated.

It is thus conceivable that determination of serum antibody titers to potentially pathogenic bacteria might be useful throughout diagnosis and treatment of periodontal patients. Such antibody studies may be used to aid in diagnosis of the particular disease entity one encounters. Likewise, these tests might be used to screen relatives of individuals with LJP or other more rapidly destructive diseases in hopes of preventing or minimizing periodontal destruction. Antibody analysis could be used to determine the therapeutic modalities to be utilized in treating a particular disease entity. Determination of specific antibody responses at various intervals throughout the course of periodontal therapy might then suggest alterations in the planned treatment. Finally, use of antibody analysis during the maintenance phase of therapy might suggest the need for further treatment.

F. Immunologic Assays

Prior to the 1970s, the most widely accepted assays employing antibodies and antigens were immunofluorescence and radio-immunoassay. In immunofluorescence, a fluorescent dye is conjugated to the antibody, while in radio-immunoassay, a radioactive isotope acts as the label (Voller *et al.* 1976a). Radio-immunoassay is a highly sensitive technique which permits precise quantitation of antibody or antigen in

a fluid. However, this technique requires complex equipment and the radioisotopes may decay quickly. Furthermore, because of the medical hazards of radiation, the materials require special handling by highly trained personnel, making this technique impractical in a clinical setting. Immunofluorescence does not possess the potential hazards associated with radio-immunoassay. Yet in practice, immunofluorescence is difficult to quantify for antibody studies because it depends on the subjective visual assessment of fluorescence. Hence, the results of immunofluorescent analysis are usually expressed as the serial dilution of serum that produces a predetermined level of fluorescence. This technique also involves use of specialized equipment, limiting its clinical applicability.

Engvall & Perlmann (1972) introduced enzyme immunoassays as an alternative to immunofluorescence and radio-immunoassay. They used polystyrene tubes as a solid phase to which antigens were adsorbed. The antigen-coated tubes were then incubated with rabbit antisera. After washing, the tubes were incubated with sheep anti-rabbit IgG which was conjugated to the enzyme alkaline phosphatase. After excess conjugate was washed out of the tubes, the amount of alkaline phosphatase bound to the tubes was determined by using p-nitrophenylphosphate (NPP) as a substrate. This results in a yellow color change which can then be measured in a spectrophotometer. The authors recommended incubation times of 6 hours for the primary antisera and 8 hours for the enzyme-conjugated secondary antibody.

Different carrier surfaces have been used for ELISA including beads, tubes, and plates. Sepharose beads permit covalent linkage of antigen or antibody to the surface, yet their large-scale use is limited (Voller *et al.* 1976a). More suitable for large-scale use are polystyrene tubes (Engvall & Perlmann 1972) or microtiter plates (Ebersole *et al.* 1980c). The plates are especially useful because they are inexpensive and small amounts of reagents can be used.

Enzyme immunoassays have been used in a wide variety of ways. Van Weemen & Schuurs (1971) measured human chorionic gonadotropin (HCG) from urine of pregnant and non-pregnant females using horseradish peroxidase as an enzymatic label rather than using radioactive iodine as previously done in radio-immunoassay.

They found the enzyme immunoassay to be highly sensitive in detecting HCG and suggested that such as assay might be applied to wide-scale clinical testing of urine samples. These authors likewise found this assay to be highly sensitive and specific for detecting a number of estrogen compounds (Van Weemen & Schuurs 1972).

The ELISA has been used in immunopathology to quantify immunoglobulins (Engvall & Perlmann 1972, Hoffman 1973). Pesce *et al.* (1974) used this enzyme immunoassay to measure DNA antibodies in lupus erythematosus. Portsmann *et al.* (1985) utilized the technique to detect and quantify alpha-1-fetoprotein.

Enzyme immunoassays have received widespread application in measurement of antibodies in infectious diseases. Carlsson *et al.* (1972) were the first to use an ELISA in this manner. They utilized the technique to detect antibodies against O antigens derived from the lipopolysaccharide of *Salmonella* species. The authors found ELISA to be a highly sensitive and specific method for detecting these antibodies. Importantly, the ELISA was able to detect IgG and IgM antibodies with equal efficiency. This differed from previously used tests in which the sensitivity for IgM antibodies was 10-100 times greater than for IgG. Similar assays have been used for detecting antibodies to *Escherichia coli* (Jodal *et al.* 1974). Voller *et al.* (1976a) have utilized the technique in serology studies of syphilis, rubella, encephalitis, herpes simplex, measles, cytomegalovirus, and hepatitis B. Voller *et al.* (1974, 1975) used an ELISA in an epidemiologic study of malaria. They also applied ELISA to diagnosis of toxoplasmosis (Voller *et al.* 1976b). In general, these studies have shown that enzyme immunoassays are equally or more sensitive than previously used serological tests and are much easier to perform.

Enzyme immunoassays have been applied extensively to serological studies of patients with periodontal disease. The previously mentioned studies assessing serum antibody levels to oral bacteria have primarily utilized the ELISA technique. However, radio-immunoassay (Tew *et al.* 1985) and immunofluorescence (Listgarten *et al.* 1981, Zambon *et al.* 1983a) are still in use in some laboratories. Ebersole *et al.* (1980c) modified the technique of Engvall & Perlmann (1972) for use in detecting serum IgG antibodies to *A.actinomycetemcomitans*. Antigens are placed in polystyrene microtiter

plates and incubated with experimental sera for 2 hours. This is followed by incubation with rabbit anti-human IgG antisera for 2 hours then alkaline phosphatase-conjugated goat anti-rabbit IgG for 16-18 hours. Addition of substrate results in a yellow color change which is then measured quantitatively in a spectrophotometer. In comparing the ELISA to immunofluorescence and passive hemagglutination, the authors found the ELISA to be 5-50 times more sensitive in detecting antibody to *A.actinomycetemcomitans*. The use of microtiter plates allowed utilization of small volumes of reagents, making this technique especially useful for rapid screening of large numbers of human serum samples for antibodies to many intact bacterial organisms. In addition to IgG antibodies, this technique has been applied successfully to measurement of IgM (Ebersole *et al.* 1979) and IgA antibodies (Ebersole *et al.* 1978).

The ELISA has also been used in microbiologic analysis of dental plaque samples in a procedure known as "serological ELISA". Ebersole *et al.* (1984b) prepared antibodies to various species of *Bacteroides* in rabbits and conjugated horseradish peroxidase to these antibodies. They then placed suspensions of *Bacteroides* species in microtiter plates and added the enzyme-conjugated antibodies in an effort to identify the particular organisms. The authors found the serological ELISA to be a rapid and reproducible means of identifying *Bacteroides* species. The technique was able to identify the organisms within 2 hours. Furthermore, it was capable of identifying specific *Bacteroides* species in mixtures of numerous organisms. More recently, the serological ELISA has been expanded to enable identification of a wide variety of oral organisms (Ebersole *et al.* 1987b). Thus, the technique is a sensitive and specific means of identifying bacteria frequently found in the subgingival plaque of patients with periodontal disease.

C. Use of Nitrocellulose

In 1975, Southern revolutionized the study of the structure and sequence of deoxyribonucleic acid (DNA) when he introduced the use of nitrocellulose as a solid phase binding material in nucleic acid research. Polyacrylamide gel electrophoresis had been used as a standard tool for analyzing and purifying DNA fragments and

proteins, yet immobilization of these compounds was not possible. Southern fractionated DNA and separated the fragments by electrophoresis in agarose or polyacrylamide gels. The DNA fragments were then immobilized by blotting them onto a sheet of nitrocellulose. The immobilized DNA was analyzed by *in situ* hybridization usi g a radioactive complimentary ribonucleic acid (RNA) label. The fragments of DNA containing transcribed nucleic acid sequences were detected as sharp bands by autoradiography.

Kafatos *et al.* (1979) modified the Southern blotting technique in a procedure known as "dot hybridization". Multiple samples of cloned DNA were spotted next to one another on a sheet of nitrocellulose paper. The nitrocellulose was then hybridized with a radioactive probe containing a DNA or RNA mixture possessing complimentary nucleic acid sequences. Following autoradiography, the extent of hybridization with each of the DNA spots on the nitrocellulose was evaluated semi-quantitatively by visual comparison with a standard dilution series of radioactive DNA similarly spotted on a sheet of nitrocellulose. This dot hybridization allowed for rapid, convenient evaluation of a large number of nucleic acid sequences in a mixture.

Renart *et al.* (1979) first reported the transfer of proteins from polyacrylamide/agarose gels to diazobenzyloxymethyl (DBM) paper in order to immobilize the proteins. After transfer, specific proteins were detected by autoradiography using a radioactive label. They found the efficiency of transfer to be 11-16%, with 21-60% of the proteins left in the gel. While this efficiency of transfer was not outstanding, it was sufficient to allow detection of as little as 100 picograms of antigen.

Bowen *et al.* (1980) demonstrated a similar technique using nitrocellulose instead of DBM paper. They used a radioactively labeled probe to analyze proteins previously separated on polyacrylamide gels and transferred to nitrocellulose. In contrast to Renart *et al.* (1979), these authors were able to successfully transfer 75% of the proteins using nitrocellulose.

Landmark research by Towbin *et al.* (1979) first demonstrated immunologic analysis of proteins transferred from polyacrylamide gels to nitrocellulose. Proteins

separated by gel electrophoresis were transferred to nitrocellulose, resulting in a replica of the pattern from the gels. This procedure immobilized the proteins so they could be examined immunologically. Some denaturation of proteins occurred during transfer. However, it is now known that any adverse effect derived from this denaturation can be minimized by renaturing the proteins with an appropriate detergent (Wedege *et al.* 1988). The residual binding capacity of the nitrocellulose sheets was blocked with bovine serum albumin. This was followed by incubation of the sheets with an appropriate primary antiserum, then with a secondary antibody specific for the first. This second antibody was either radioactively labeled or conjugated with fluorescein or horseradish peroxidase. The specific proteins being analyzed could then be identified by autoradiography, by ultraviolet light, or by the peroxidase reaction product. The authors found the radioactively labeled and peroxidase-conjugated antibodies to be much more sensitive that the fluorescein-conjugated antibodies. Importantly, they found the peroxidase technique to be highly sensitive in detecting very small amounts of antibody in sera of low titer.

The production of monoclonal antibodies has dramatically improved immunologic procedures (Kohler & Milstein 1975). Hybridization between myeloma cells and antibody-secreting cells has become a useful tool for producing large quantities of monoclonal antibodies directed against specific antigens. Sharon *et al.* (1979) used nitrocellulose to detect specific monoclonal antibodies secreted by hybridomas. Nitrocellulose sheets were incubated with specific antigens and then incubated with antibody-producing hybridomas growing in agar. If antibodies specific for these antigens were being produced by the hybridomas, they were easily detected.

Hawkes *et al.* (1982) described the use of nitrocellulose for analyzing antigen-antibody reactions in a technique called "dot-immunoblotting". In this procedure, a diluted antigen solution is "dotted" onto a sheet of nitrocellulose and then incubated first with the experimental antibody and then with a peroxidase-conjugated secondary antibody directed against the first. After developing the peroxidase, a positive reaction is detected as a colored dot on the nitrocellulose. These authors have tested soluble proteins, nucleic acids, membranes, fungi, viruses, and a number

of other antigens, all of which have been shown to bind tightly to the nitrocellulose. They have used the technique to assay large numbers of samples for the presence/absence of monoclonal antibodies. Furthermore, they have used dot-immunoblotting to analyze human sera for antibodies to a variety of pathogens, demonstrating the versatility of the technique as a diagnostic tool. The authors feel that this procedure has two distinct advantages over enzyme immunoassays that employ microtiter plates. First, the amount of antigen needed for dot-immunoblotting is extremely small. A visible dot 0.3mm in diameter was present on the nitrocellulose with as little as 0.1 µl of sample. Larger volumes of sample simply increases the size of the spot. Second, using nitrocellulose allows one to visualize the color change against a white background, making it easier to detect positive reactions and to reject false positives.

Heberling & Kalter (1986) used a similar technique, which they called "dot-immunobinding", for rapid detection of viral antigens and antibodies. They tested 680 human and nonhuman primate sera for antibodies to several viruses. The overall sensitivity of the dot-immunobinding assay was 97.8%. The assay was also found to be extremely sensitive in detecting viral antigen in the sera. The authors conclude that dot-immunobinding could be used to rapidly identify unknown viral isolates and to detect specific viral antigens in clinical specimens, especially with the advent of monoclonal antibodies. Likewise, dot-immunobinding could be utilized for rapid identification of serum antibodies to specific viruses, allowing swift diagnosis and initiation of treatment.

Heberling & Kalter (1985, 1987) used the dot-immunobinding assay to test 150 macaque monkey sera for antibodies to *Herpesvirus simiae*, a monkey virus which has the potential to cause fatal infection in humans. Sera were also tested by serum neutralization, the most commonly used laboratory test for viral antigen testing prior to this report. Of the 150 sera, 83 were negative to both serum neutralization and dot-immunobinding, while 56 were positive to both. Eleven sera were positive by dot-immunobinding but negative to serum neutralization. Thus, using serum neutralization as the "gold standard", dot-immunobinding detected antibody to the

virus with a sensitivity of 100% and a specificity of 88%. The dot-immunobinding assay was much more rapid than serum neutralization, with results obtained in one day by the former versus three to five days by the latter. The authors conclude that dot-immunobinding is a rapid and simple test for detecting serum antibody to the *Herpesvirus*, with excellent sensitivity and specificity. Moreover, they suggest that this assay could easily be applied to detection of other viral antibodies from clinical specimens.

Nitrocellulose has been used to detect and quantify influenza viruses (Furuya *et al.* 1984). Purified influenza virus suspensions were passed through a filtration device containing a nitrocellulose filter. The nitrocellulose was then treated with mouse anti-influenza antibody followed by peroxidase-conjugated sheep anti-mouse igG antibody. The color change was noted and recorded in a semi-quantitative/qualitative manner (negative, positive, strongly positive). In addition, quantification of the blue-colored dots was determined by visual adsorption in a spectrodensitometer. The authors also used this technique to detect influenza viruses from clinical throat swab specimens. They conclude that the assay is ideally suited to detection of respiratory viruses in a clinical setting.

Talbot *et al.* (1984) found that dot immunoblotting with nitrocellulose was as sensitive as radio-immunoassay and ELISA in detecting very low levels of viral antigen. Likewise, the technique was able to detect extremely small amounts of monoclonal antiviral antibodies produced by hybridomas. Specifically, a 48,600-fold dilution of antiviral antibody still gave a distinct signal (colored dot) with the dot immunoblotting assay, compared to an end-point titer of 1:18,000 with ELISA. Bode *et al.* (1984) described a technique called nitrocellulose-enzyme-linked immunosorbent assay (NC-ELISA) in which nitrocellulose disks are placed in the bottom of the wells of standard polystyrene microtiter plates. Viral antigens are then spotted onto the disks, followed by blocking of further binding capacity with bovine serum albumin. The disks are then treated by sequential incubation with rabbit antiviral antibody and alkaline phosphatase-conjugated anti-rabbit IgG. Substrate is added to produce a red color change and the degree of change is expressed on a relative scale from pale to dark

red. A conventional ELISA was also performed in order to compare results. The only difference between the NC-ELISA and the standard ELISA is the difference in solid phase support (nitrocellulose versus polystyrene). The NC-ELISA demonstrated 8- to 10-fold greater sensitivity for detection of viral antigens compared to conventional ELISA, while specificity was similar. Likewise, the NC-ELISA was extremely sensitive in detecting antiviral antibodies. The authors conclude that the greater sensitivity of the NC-ELISA relative to the ELISA is due to the better binding capacity of nitrocellulose compared to polystyrene. Thus, in addition to the advantages over the ELISA described by Hawkes *et al.* (1982), the dot-immunoblotting type of assay has the added benefit of greater antigen binding, with the consequent ability to use smaller concentrations of antigen.

While much of the experimentation utilizing nitrocellulose has been in the fields of virology and nucleic acid research, this material has also been used for isolation of specific organisms in vaccine development (Lu *et al.* 1988). It has been employed in diagnosis of other non-viral infectious diseases as well. Angeles & Sugar (1987) use a dot-blotting assay to detect IgG antibody to several species of *Nocardia* in an attempt to ease diagnosis of the disease. They found that 100% of sera from patients with confirmed nocardiosis had a positive reaction, while 100% of non-infected controls were negative.

Nitrocellulose has also been used in research in the dental field. O'Lee & Boackle (1987) used the material to analyze the interaction of non-immunoglobulin salivary agglutinins and human salivary IgG and to assess their effects on agglutination of oral bacteria. McArthur *et al.* (1986) applied nitrocellulose in the field of periodontology. Specifically, they used nitrocellulose in combination with monoclonal antibodies to detect the putative periodontopathic organism *A.actinomycetemcomitans* and to differentiate its three serotypes. The organisms were placed in culture dishes for 4 days. Circular nitrocellulose disks were placed in the dishes for 15 minutes to passively blot the bacterial colonies onto the material. The disks were then incubated with murine hybridoma-derived monoclonal antibodies specific for each of the three serotypes. Subsequent incubation with peroxidase-conjugated anti-murine IgG and

substrate led to identification of the different serotypes. Plaque samples were taken from a patient with juvenile periodontitis and from squirrel monkeys with naturally-occurring periodontitis. The samples were treated as described. Results demonstrated that when *A.actinomycetemcomitans* was detected by gram-staining, growth in selective culture media, and ELISA, the organism was also detected by the nitrocellulose assay 99-100% of the time. Thus, the assay was able to accurately detect *A.actinomycetemcomitans* in plaque samples and characterize the organisms as to serotype. The authors suggest that this type of assay could greatly simplify the identification and possibly the quantitation of organisms from subgingival plaque samples.

H. Enzyme Substrate Systems

Various enzyme conjugates are available for use in enzyme immunoassays including alkaline phosphatase, horseradish peroxidase, and β-galactosidase. In general, it appears that alkaline phosphatase is preferred by most authors when using the ELISA, most likely due to its high activity and bright yellow reaction color which can be assessed visually or spectrophotometrically (Engvall & Perlmann 1972, Ebersole *et al.* 1980c, Mouton *et al.* 1981, Chung *et al.* 1983, Naito *et al.* 1984, Genco *et al.* 1985, Haffajee *et al.* 1988, Aukhil *et al.* 1988). Horseradish peroxidase also has high activity, and is generally less expensive than alkaline phosphatase. Peroxidase has been shown to be an excellent enzyme for conjugation (Nakane 1975). Most studies utilizing nitrocellulose have preferred horseradish peroxidase as the enzyme conjugate (Towbin *et al.* 1979, Hawkes *et al.* 1982, Furuya *et al.* 1984, McArthur *et al.* 1986, Angeles & Sugar 1987, O'Lee & Boackle 1987). However, alkaline phosphatase has also been used (Bode *et al.* 1984, Heberling & Kalter 1986, 1987). β-galactosidase is infrequently used in ELISA and dot-blotting assays. Portsmann *et al.* (1985) examined the use of alkaline phosphatase, horseradish peroxidase, and β- galactosidase in an enzyme immunoassay for detection of alpha-1- fetoprotein. The peroxidase-IgG conjugates provided the lowest detection limits for this protein, while alkaline phosphatase and β-galactosidase required greater protein concentrations for detection. Peroxidase also demonstrated the highest sensitivity at the shortest period of reaction

time. During a period of 30 minutes, peroxidase conjugates had the highest level of chromogenic substrate conversion with the smallest amount of alpha-1- fetoprotein. Voller *et al.* (1976a) believe the choice of enzyme for conjugation is primarily a matter of personal preference.

The substrate used depends upon the enzyme chosen for conjugation. Most authors using horseradish peroxidase as the enzyme conjugate in dot-blot assays prefer a substrate consisting of 4-chloro-1-naphthol/ Tris-buffered saline/ methanol/ hydrogen peroxide. This combination results in a blue to purple color change (Hawkes *et al.* 1982, McArthur *et al.* 1986, Angeles & Sugar 1987, O'Lee & Boackle 1987). When alkaline phosphatase is used in dot-blot assays, the substrate is usually naphthol AS-MX phosphate and Fast Red TR Salt, which produces a red color (Heberling 1986, 1987). When alkaline phosphatase is used for ELISA, the substrate is usually p-nitrophenylphosphate, which produces a distinct yellow color change (Engvall & Perlmann 1972, Ebersole *et al.* 1980c, Portsmann *et al.* 1985).

STATEMENT OF PURPOSE

The goal of this research was to design a simple, rapid, qualitative assay for determining, in a clinical setting, the presence of elevated serum antibody levels to potentially periodontopathic microorganisms. This information might be particularly useful in clinical research involving large subject populations. Likewise, it might be beneficial in large-scale epidemiologic studies. However, the primary intention was to design an assay which could be used by the clinician as an aid in diagnosis and treatment of individual periodontal patients.

An ideal assay for use in such a clinical setting would have the following characteristics:

1. Simple, relatively pain-free method for collecting antibody-containing sample.
2. Minimal time from sample collection to determination of results; preferably a matter of minutes to hours, rather than days to weeks.
3. Minimal number of reagents required.
4. Minimal amount of specialized equipment and space.
5. Simple, repeatable technique.

6. Minimal training required; can be performed by auxiliary personnel.
7. Capable of detecting elevated antibody levels to organisms of interest.
8. Inexpensive

The overall objective of this research was to develop an assay with as many of these characteristics as possible.

II. MATERIALS AND METHODS

A. Serum Versus Capillary Blood Antibody Titers

1. Collection of Samples.

In order to develop a rapid and inexpensive assay, the use of whole peripheral capillary blood rather than venous-derived serum was preferred. This reduces time and equipment needed to separate the serum from other blood components. Venous blood was collected from ten human volunteers of undetermined periodontal status. The sera were separated by centrifugation after clotting and stored until use at -20°C. Heparin-coated capillary tubes (Chase Instruments Cat. No. 501; internal diameter 1.1-1.2 mm; length 75mm) were marked such that a volume of 60 µl could be accurately collected. Sixty microliters of peripheral capillary blood was obtained from each subject via finger-stick and drawn into the capillary tubes. The blood was then expressed into phosphate-buffered saline (PBS) containing 0.05% Tween 20 to obtain a dilution of 1:50. These samples were either used immediately or stored at -20°C until use.

2. Enzyme-linked Immunosorbent Assay.

Antibody levels in serum and capillary blood were assessed by enzyme-linked immunosorbent assay (ELISA) as described by Ebersole *et al.* (1980c). Whole formalinized bacteria were used as antigens, specifically *A.actinomycetemcomitans* strain Y4, *B.gingivalis* ATCC 33277, and *B.intermedius* ATCC 25611.

Whole formalinized bacteria were diluted in buffer at pH 9.6 containing 0.2M sodium carbonate (Na_2CO_3), 0.2M sodium bicarbonate ($NaHCO_3$), and 10% sodium azide. Ninety-six well polystyrene microtiter plates (Linbro Co.) were coated with various dilutions of whole bacteria and incubated at 37°C for 3-4 hours. Plates were then stored at 4°C until use.

Antigen-coated plates were washed three times (5 minutes each) with 0.9% sodium chloride (NaCl), pH 7.4 containing 0.05% Tween 20. Plates were then incubated at room temperature for 2 hours with serum and capillary blood samples diluted to 1:50 in PBS/Tween 20. Following three 5 minute washes with

NaCl/Tween 20, the plates were incubated with a 1:500 dilution of affinity purified goat anti-human IgG (Calbiochem #401441) at room temperature for 2 hours.

After washing, the plates were incubated overnight (16-18 hours) with a 1:1000 dilution of alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma #A7650; heavy and light chain specific, affinity purified). Following a final washing, p-nitrophenyl phosphate (Sigma 104 Phosphatase Substrate; 1 mg/ml in pH 9.8 buffer containing Na_2CO_3, $NaHCO_3$, and 1mM $MgCl_2$) was added to the plates as substrate. The reactions were terminated at 30 minutes by addition of 1N NaOH. The absorbance at 410 nm was then determined using an ELISA reader (Dynatech MR 650) and Immunosoft computer software (Dynatech Immunosoft 2.5).

Each serum and capillary blood sample was assayed in triplicate and a mean titer determined. Antibody activities in the samples were related to a standard reference serum curve for each organism. The serum standard was assigned a reactivity of 100 ELISA units (EU) and a reference curve relating optical density to the $\log_{10}$ EU was produced for each microtiter plate.

Antibody reactivity in serum and capillary blood was compared for each subject. The percentage of reactivity in capillary blood relative to the reactivity in serum was then determined.

B. Development of Dot-Blot Assay

1. Selection of Enzyme-substrate System.

The initial step in developing the dot-blot assay was the determination of an appropriate enzyme-substrate system. The chromogenic system selected had to produce a distinct color change and yet require a relatively short reaction time. Three enzymes were selected for examination: alkaline phosphatase, horseradish peroxidase, and β-galactosidase. An experiment was designed on nitrocellulose paper (Schleicher & Schuell Cat. No. 41-00850; pore size 0.45 μm) in which various antigen concentrations, serum dilutions, and enzyme dilutions could be analyzed in order to obtain an ideal concentration of each reagent to be used in the final assay.

Immunoblotting on nitrocellulose paper involves "spotting" diluted antigens onto the paper then reacting these "dots" with antibody-containing fluid. The

volume of antigen suspension varies, but readable results have been obtained with volumes of 0.1 - 20 µl, depending on the concentration of the antigen (Hawkes 1982, Heberling & Kalter 1986, 1987). Whole formalinized bacteria were diluted in PBS to obtain concentrations of 1×10^5 to 1×10^9 organisms per 10 µl drop of solution. Nitrocellulose sheets were wetted by placement in a plastic dish (Tupperware) containing Tris-buffered saline (TBS). Whatman chromatography paper (Whatman Cat. No. 3030Y917; thickness 0.34 mm) was placed in the bottom of another plastic dish and moistened with TBS. The nitrocellulose was then removed from the TBS and placed on top of the Whatman paper in order to keep the nitrocellulose moist. After excess TBS had absorbed from the nitrocellulose into the Whatman paper, the diluted antigens were "spotted" onto the nitrocellulose sheets and allowed to dry. Once the "dots" were no longer visible, the nitrocellulose sheets were hung to dry for 30 minutes. Drying at this stage will stabilize the binding of the bacteria to the nitrocellulose; in fact, the sheets can be stored at this stage for several weeks without loss of activity (Hawkes 1982). Non-specific binding sites were then blocked by submerging the nitrocellulose in various concentrations of nonfat dry milk (Sanilac) in TBS. After blocking, the nitrocellulose sheets were rinsed thoroughly in TBS. The sheets were then either dried and stored or used immediately.

The reference standard sera for each organism used in the ELISA were diluted to 1:50. Small strips of filter paper (Whatman Cat. No. 3001Y861; thickness 0.18 mm) were cut and wetted thoroughly with serum. New pieces of Whatman chromatography paper were placed in clean, dry plastic ware and moistened with TBS. After the nitrocellulose sheets were placed on the Whatman paper, the serum-containing strips were laid over the antigen "spots" in order to allow incubation. The plastic dishes were covered tightly for two hours. The serum-strips were then removed and the nitrocellulose was washed thoroughly with TBS. At this stage, the nitrocellulose was incubated with horseradish peroxidase-conjugated anti-human IgG (Cappel Cat. No. 3201-0081) at various dilutions in TBS. After washing the nitrocellulose in TBS, the sheets then were

placed in a dish containing 20 ml of methanol, 60 mg of 4-chloro-1-naphthol (Sigma No. C-8890), 0.6 ml of 3% hydrogen peroxide, and 100 ml of TBS as substrate. When the reactions were terminated, the degree of bluish-purple color change for each "dot" could be assessed.

Preliminary evaluation of β-galactosidase as a chromogenic enzyme revealed the need for prolonged incubation of up to 12 hours. This was deemed inappropriate for the assay being developed. Alkaline phosphatase had a short reaction time and distinct color change, similar to the properties of horseradish peroxidase. The majority of studies utilizing nitrocellulose have preferred horseradish peroxidase as the conjugate (Towbin *et al.* 1979, Hawkes *et al.* 1982, Furuya *et al.* 1984, McArthur *et al.* 1986, Angeles & Sugar 1987, O'Lee & Boackle 1987). Peroxidase is generally less expensive than alkaline phosphatase as well. The choice of horseradish peroxidase for use in developing this assay was, therefore, primarily one of availability, cost and personal preference.

2. Determination of Appropriate Antigen, Serum and Reagent Dilution.

Once an adequate chromogenic enzyme was selected, attention was focused on determining the appropriate serum dilution and antigen concentration to be used. From the ten individuals previously examined, a high reactive and a low reactive serum sample for each organism was selected. The dot-blot assay was performed with antigen concentrations varying from 1×10^5 to 1×10^9 organisms per 10 µl drop and serum dilutions ranging from 1:10 to 1:50. In order to determine which dilutions provided the best distinction between high and low responders, the high and low reactive sera at any given dilution were placed side-by-side on the nitrocellulose. The serum dilution which allowed the best distinction between positive and negative reactions to all three organisms was then chosen. This would enable one to perform the assay utilizing a single dilution of antibody-containing fluid to assess reactivity to all organisms.

Following selection of an appropriate serum dilution, assays were performed to further narrow, and finally select, the appropriate antigen concentration for each individual organism. From a pool of serum samples with known antibody reactivity

to the bacteria being tested, six high responders and six low responders were chosen for each organism. The dot-blot assay was accomplished on multiple occasions in order to determine, for each organism, the concentration which best delineated a positive from a negative response. Concentrations ranged from 5 x 10^5 to 1 x 10^8 bacteria per 10 µl drop, with each successive assay further narrowing the concentration range. The concentration of powdered milk used for blocking non-specific nitrocellulose binding sites was varied from 1% to 5%. The degree of non-specific color change outside the antigen "dots" but within the area covered by the serum-containing strips was assessed for each concentration.

Finally, the concentration of enzyme-conjugated anti-human IgG was varied from 1:1000 to 1:3000. The effect on intensity of color change was evaluated.

3. Alterations in Incubation Times.

The purpose of this research was to develop a rapid assay for determining elevated antibody to oral microorganisms. As such, incubation periods for each step in the test were varied to achieve the minimum time which would still allow for accurate results. Blocking of non-specific binding sites with powdered milk, incubation of the antigen "dots" with the serum-containing strips, and reaction of the nitrocellulose with the enzyme-conjugated anti-human IgG were varied from 30 minutes to 2 hours. Finally, reaction of the enzyme substrate ranged from one to five minutes.

With each variation in incubation time, the effects on the ability to correctly distinguish elevated from normal antibody titers was assessed.

C. Initial Results of Dot-blot Assay

Thirty-nine serum samples were selected to investigate the ability of the dot-blot assay to correctly assess the antibody response to *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius*. An ELISA was performed to determine serum antibody titers to each organism. The dot-blot assay was then performed for each sample. Nitrocellulose sheets were designed such that a single serum-containing filter strip for each subject could be placed over three antigen 'dots', one for each organism. Each nitrocellulose sheet had five to ten experimental samples plus a positive and negative

control. The positive control serum was obtained from a subject who had, in previous experiments, demonstrated elevated antibody titers to all three organisms. Likewise, the negative control serum had previously shown normal antibody reactivity to all the bacteria. Each nitrocellulose sheet possessed its own positive and negative control for direct comparison with experimental samples.

After the enzyme-substrate reaction, the degree of color change for each "dot" was assessed and the reaction was deemed either positive or negative relative to control "dots". The qualitative results of the dot-blot assay were then compared in a blind fashion to the quantitative antibody titers as determined by ELISA. The ability of the dot-blot assay to correctly identify subjects with elevated versus normal antibody responses to the three organisms was then ascertained.

D. Use of Assay in Screening of Patients

Thirty-four patients with various forms of periodontal disease were selected at random from the Postdoctoral Periodontics Clinic at the University of Texas Health Science Center at San Antonio, Texas. Venous blood was drawn and allowed to clot. Digi-puncture was performed and 60 µl of peripheral capillary blood was drawn into heparinized capillary tubes, followed by dilution to 1:25 in PBS/Tween 20. Serum was separated from venous samples by centrifugation. Serum and capillary blood samples were then stored at -20°C until use.

The ELISA was performed in triplicate for each serum and capillary sample to assess quantitative antibody titers to *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius*. For ELISA, 1:100 dilutions of serum and capillary blood were used. The proportion of reactivity in capillary blood relative to serum was also determined for each patient.

The dot-blot assay was then performed. The appropriate antigen "dots" were placed on the nitrocellulose sheets. The sheets were dried and blocked with powdered milk. Approximately 300 µl of the 1:25 dilution of capillary blood was placed in a 15 ml test tube marked by code for each subject. Strips of Whatman filter paper large enough to cover the three antigen "dots" (about 1 x 4 cm) were placed in the test tubes. Nitrocellulose sheets were placed in the bottom of plastic dishes on

top of previously moistened chromatography paper. After the blood-containing strips were thoroughly wet, they were removed from their coded test tubes and laid over the column of three antigen "dots" with the corresponding patient code. The dishes were then covered and allowed to incubate. After appropriate incubation time, the blood-containing strips were removed and discarded. The nitrocellulose sheets were then incubated with the peroxidase-conjugated anti-human IgG and developed with substrate.

Each "dot" was graded as either elevated or not elevated based on the degree of color change relative to positive and negative controls on the same nitrocellulose sheet. This was done in a blind fashion, as the examiner was unaware of the results of the ELISA. The qualitative positive and negative results from the dot-blot were then compared to the respective quantitative ELISA results for each subject.

Similar experiments were performed utilizing serum and capillary blood from 10 periodontally healthy subjects. Again, ELISA and dot-blot results were analyzed for agreement relative to elevation of antibody titers to these organisms.

E. Statistical Analysis

Antibody titers in serum and capillary blood were compared for each subject and for the population as a whole. Mean serum titers for each organism were analyzed and compared to mean capillary blood titers. The results were expressed as the percent reactivity in capillary blood relative to serum. The correlation between serum and capillary blood titers to each organism was determined by regression analysis and analysis of variance. Spearman rank correlations between serum and capillary blood titers were also determined for each organism. Capillary and serum titers to the three organisms were paired for each subject and analyzed by Wilcoxon Paired Ranks Test to determine the relationship between values for each individual rather than comparing means of values for the entire population.

In order to determine the appropriate dilution of capillary blood to be used in the dot-blot assay, capillary antibody titers were adjusted by factors related to the relative reactivity in capillary blood versus serum. Adjusted capillary titers were compared to measured capillary ELISA titers by Wilcoxon Paired Ranks Test.

Contingency tables were designed to evaluate the sensitivity and specificity of the dot-blot assay relative to the ELISA for the 39 initial serum samples, for capillary blood samples from 34 periodontally diseased subjects, and for capillary blood samples from 10 periodontally healthy patients. ELISA and dot-blot values were evaluated by Fisher's Exact Test and Chi-square analysis to determine the significance of the relationship between the two assays.

Contingency tables were devised to assess the association between positive or negative dot-blot results and the presence or absence of periodontal disease. Similar tables were constructed for the ELISA results. Sensitivity, specificity, positive predictive value and negative predictive values were determined to examine the ability of both assays to predict the periodontal status of the subjects. The tables were analyzed by Chi-square and Fisher's Exact Test to assess their statistical significance.

III. RESULTS

A. Serum Versus Capillary Blood Antibody Titers

1. ELISA Results.

Antibody titers were assessed by ELISA in capillary blood and serum samples from a total of 32 subjects. These titers are represented diagrammatically in the box-and-whisker plots (Figures 1 and 2). Figure 1 represents the spectrum of serum antibody titers to *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius*. Capillary blood titers are depicted in Figure 2. As expected, there was a wide range of reactivity to the various bacteria. For each organism, mean and median titers were calculated and the standard deviation and standard error of the mean were determined. Likewise, these parameters were assessed for total mean titers to all organisms.

Reactivity in capillary blood was then compared to that in serum (Table 1). The mean serum titer to *A.actinomycetemcomitans* was 150.5 $\pm$ 205 EU compared to a mean capillary blood titer of 68.7 $\pm$ 106 EU. Serum and capillary titers to *B.gingivalis* were 19.3 $\pm$ 21.7 EU and 14.4 $\pm$ 21 EU respectively. For *B.intermedius*, mean serum titer was 111.4 $\pm$ 90 EU compared to a mean capillary titer of 50.4 $\pm$ 40.2 EU. When antibody reactivity to all organisms was analyzed, the mean serum titer was 93.7 $\pm$ 140.1 EU compared to a mean capillary titer of 44.5 $\pm$ 69.6 EU.

The level of reactivity in capillary blood was calculated as a percentage of the serum titers for the 32 subjects in whom both samples were taken. The mean percent reactivity in capillary blood was then determined for each organism and for all organisms combined (Table 2). Capillary titers to *A.actinomycetemcomitans* averaged 47.0 $\pm$ 22.2% of the serum titers. For *B.gingivalis* and *B.intermedius*, capillary antibody reactivity averaged 68.0 $\pm$ 27.4% and 48.8 $\pm$ 16.9% of the serum titers respectively. When data for all organisms were combined, capillary antibody titers averaged 54.6 $\pm$ 24.3% of the serum reactivity. There was a considerable degree of variation between individuals in the level of reactivity in peripheral capillary blood compared to serum reactivity. This is represented in the box-and

FIGURE 1.

Notched Box and Whisker representation of serum antibody levels to *Aa*, *Bg*, and *Bi*. Y-axis is serum IgG antibody reactivity in ELISA Units (EU). The box represents the middle 50% of data values, between the lower and upper quartiles (interquartile range). The notch represents the median data value for the population. The "Whiskers" extend to include data values within 1.5 times the interquartile range. The points represent "Outliers" which are sera with extremely elevated serum antibody titers.

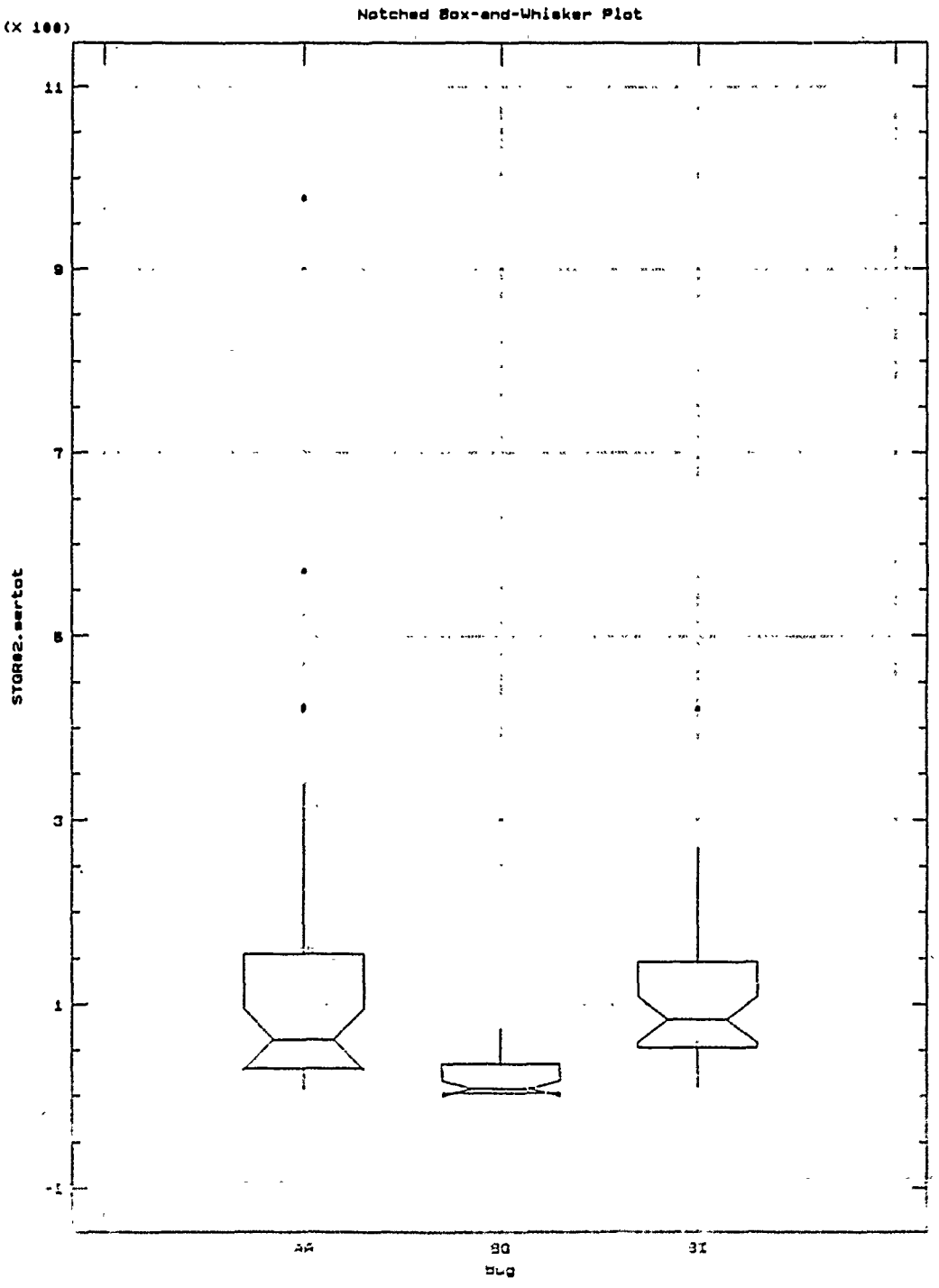

Notched Box-and-Whisker Plot
(X 100)
11
9
7
5
3
1
STOR82.mertot
AA
BO
BI
Bug

FIGURE 2.

Notched Box and Whisker representation of capillary blood antibody levels to *Aa*, *Bg*, and *Bi*. Y-axis is serum IgG antibody reactivity in ELISA Units (EU). The box represents the middle 50% of data values, between the lower and upper quartiles (interquartile range). The notch represents the median data value for the population. The "Whiskers" extend to include data values within 1.5 times the interquartile range. The points represent "Outliers" which are sera with extremely elevated serum antibody titers.

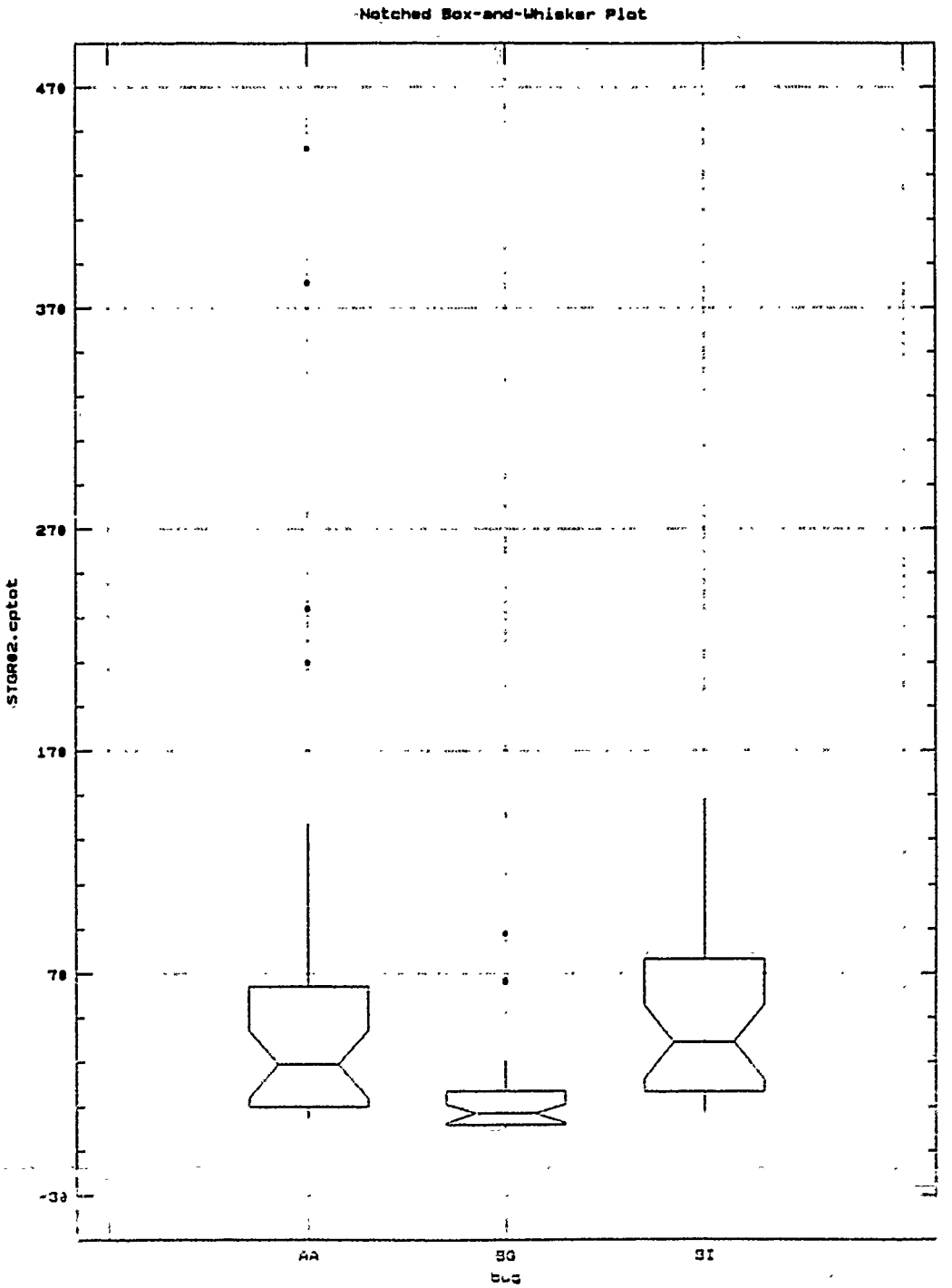

Notched Box-and-Whisker Plot
470
370
270
170
70
-30
STOR02.cptot
AA
SG
SI
bus

TABLE 1

SERUM vs. CAPILLARY ANTIBODY LEVELS as DETERMINED by ELISA

	Aa	*Bg*	*Bi*	**All Organisms**
N	*32*	*32*	*32*	*96*
Average CpEU	*68.7*	*14.4*	*50.4*	*44.5*
Median CpEU	*29*	*7*	*39*	*18*
S.D.	*106*	*21*	*40.2*	*69.6*
S.E.M.	*18.7*	*3.7*	*7.1*	*7.1*
Minimum CpEU	*5*	*0.3*	*7*	*0.3*
Maximum CpEU	*442*	*88*	*148*	*442*
N	*32*	*32*	*32*	*96*
Average SerEU	*150.5*	*19.3*	*111.4*	*93.7*
Median SerEU	*62*	*8*	*83.5*	*49*
S.D.	*205.3*	*21.7*	*90*	*140.1*
S.E.M.	*35.2*	*3.7*	*15.4*	*13.9*
Minimum SerEU	*7*	*0.5*	*9*	*0.5*
Maximum SerEU	*978*	*73*	*421*	*978*

CpEU = capillary EU by ELISA
SerEU = serum EU by ELISA
S.D. = standard deviation
S.E.M. = standard error of the mean

TABLE 2

CAPILLARY BLOOD ANTIBODY LEVELS as a PERCENT of SERUM LEVELS

	Aa	*Bg*	*Bi*	**All Organisms**
N	*32*	*32*	*32*	*96*
Average %	*47.0*	*68.0*	*48.8*	*54.6*
Median %	*46.8*	*64.4*	*48.7*	*52.2*
S.D.	*22.2*	*27.4*	*16.9*	*24.3*
S.E.M.	*3.9*	*4.8*	*3.0*	*2.5*
Minimum %	*14.3*	*25.0*	*14.5*	*14.3*
Maximum %	*104.5*	*128.6*	*80.0*	*128.6*

whisker plot relating capillary titers as a percent of serum titers (Figure 3).

The relationship between serum and capillary antibody titers was assessed by regression analysis and analysis of variance (Figures 4-7). Regression formulas were calculated for each organism and for all organisms combined. Correlation coefficients and levels of significance were then determined (Table 3). The correlation between serum and capillary blood antibody reactivity was highly significant: *A.actinomycetemcomitans* r= 0.76, *B.gingivalis* r= 0.90, *B.intermedius* r= 0.85, and all organisms combined r= 0.80. The level of significance for all correlation coefficients was $p<0.00001$. Spearman rank correlations (r_S) were also determined between capillary and serum samples (Table 4). For *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius*, respective Spearman ranks were $r_S= 0.92$, $r_S= 0.95$, $r_S= 0.92$. For all organisms combined, $r_S= 0.94$. All Spearman rank correlation coefficients were highly statistically significant ($p<0.0001$).

In order to assess the relationship between serum and capillary antibody titers in individual subjects rather than comparing mean values for all subjects, the Wilcoxon Paired Ranks Test was used to analyze the differences between paired capillary and serum titers. For *A.actinomycetemcomitans* and *B.intermedius*, serum antibody titers were significantly greater than their paired capillary titers ($p<0.005$ and $p<0.001$ respectively). While serum reactivity to *B.gingivalis* was generally greater than capillary reactivity to this organism, the difference did not reach a level of statistical significance ($p=0.194$). A total of 96 matched pairs of serum and capillary ELISA values were analyzed (34 subjects x 3 organisms per subject). Overall, serum antibody titers were statistically significantly greater than the paired capillary titers ($p<0.005$).

2. Selection of Appropriate Serum Dilution.

As discussed previously, horseradish peroxidase was chosen as the enzyme conjugate for the dot-blot assay based on its distinct color change and speed of reaction. It was necessary to determine the ideal concentration of serum to allow

FIGURE 3.

Notched Box and Whisker representation of capillary blood antibody reactivity as a percent of serum antibody levels to *Aa*, *Bg*, and *Bi*. Y-axis is serum IgG antibody reactivity in ELISA Units (EU). The box represents the middle 50% of data values, between the lower and upper quartiles (interquartile range). The notch represents the median data value for the population. The "Whiskers" extend to include data values within 1.5 times the interquartile range. The points represent "Outliers" which are sera with extremely elevated serum antibody titers.

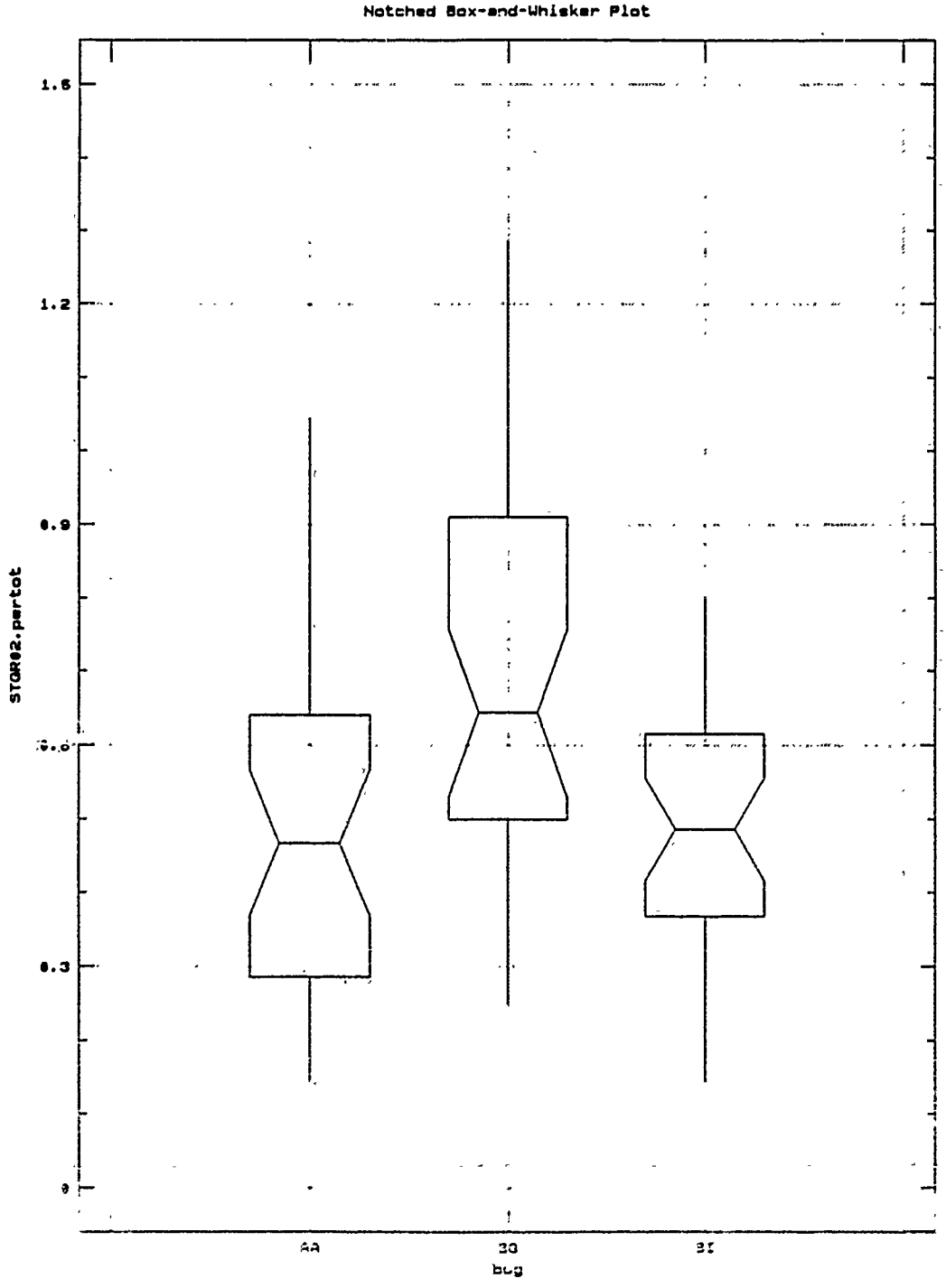

Notched Box-and-Whisker Plot
1.5
1.2
0.9
0.6
0.3
0
STOR02.partot
AA
BB
BI
bug

FIGURE 4.

Regression analysis of the relationship between serum and capillary blood antibody levels to *Aa*. The X-axis denotes serum antibody levels to *Aa* and the Y-axis denotes capillary blood antibody levels to *Aa*. The points represent individual patient levels with a group size of 32. The solid line represents a linear regression line. The inner dashed lines represent a 95% confidence interval and the outer dashed lines indicate the 99% confidence level for the regression.

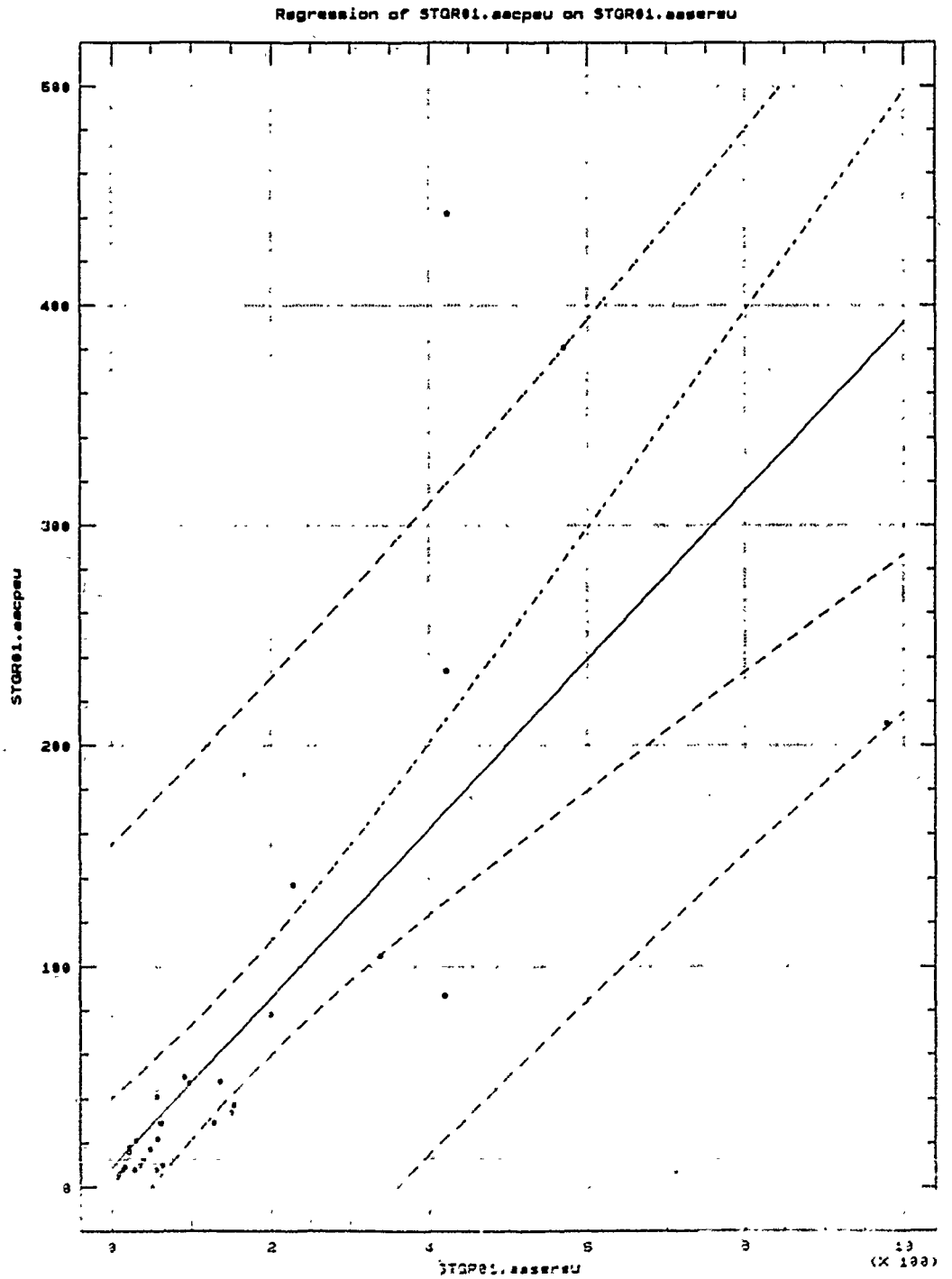

Regression of STQR01.aacpeu on STQR01.aaserau
500
400
300
200
100
0
STQR01.aacpeu
0
2
4
6
8
10
(X 100)
STQR01.aaserau

FIGURE 5.

Regression analysis of the relationship between serum and capillary blood antibody levels to *Bg*. The X-axis denotes serum antibody levels to *Bg* and the Y-axis denotes capillary blood antibody levels to *Bg*. The points represent individual patient levels with a group size of 32. The solid line represents a linear regression line. The inner dashed lines represent a 95% confidence interval and the outer dashed lines indicate the 99% confidence level for the regression.

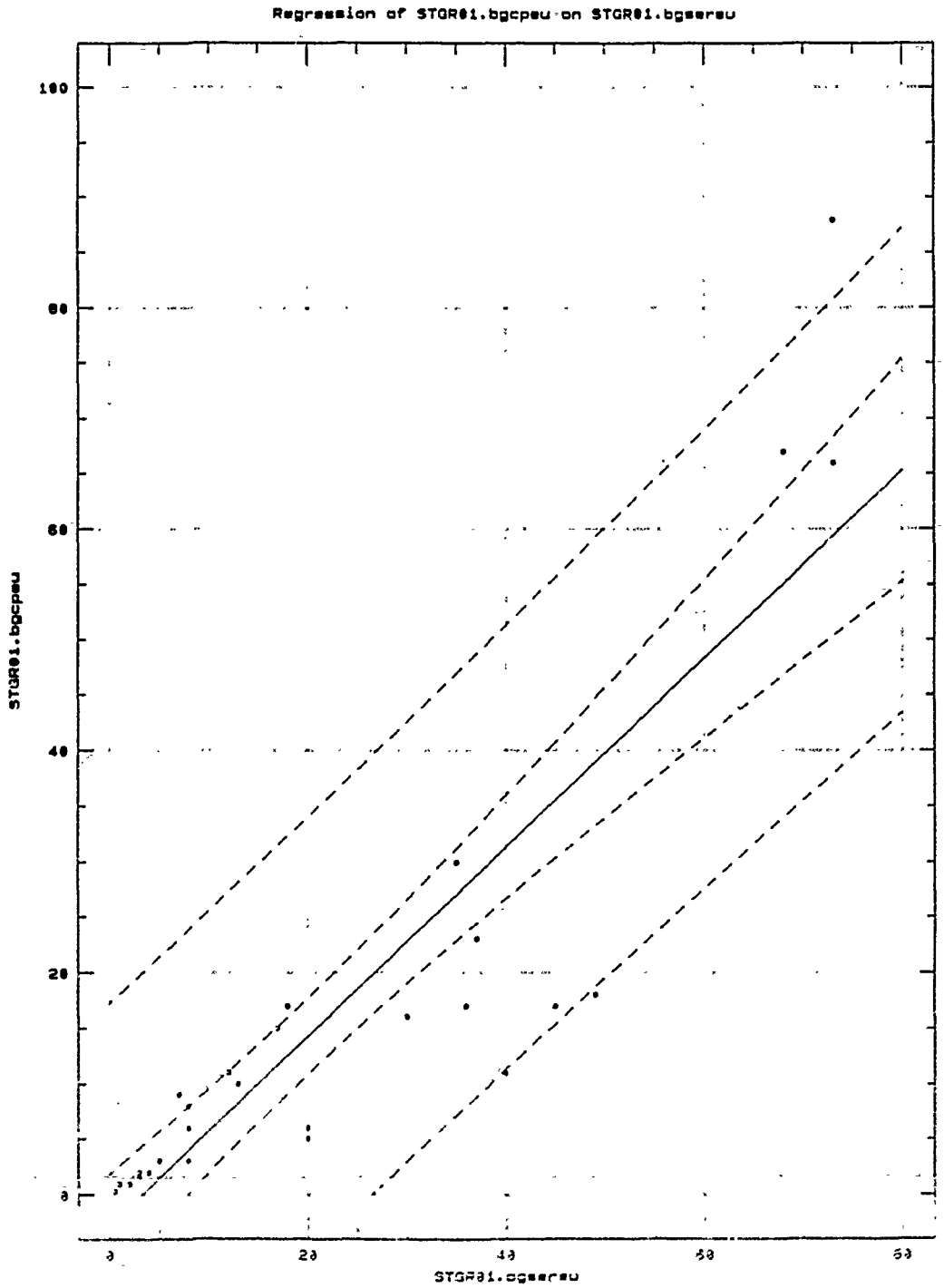

Regression of STGR01.bgcpeu on STGR01.bgseeeu
100
80
60
40
20
0
STGR01.bgcpeu
0
20
40
60
STGR01.bgseeeu

FIGURE 6.

Regression analysis of the relationship between serum and capillary blood antibody levels to *Bi*. The X-axis denotes serum antibody levels to *Bi* and the Y-axis denotes capillary blood antibody levels to *Bi*. The points represent individual patient levels with a group size of 32. The solid line represents a linear regression line. The inner dashed lines represent a 95% confidence interval and the outer dashed lines indicate the 99% confidence level for the regression.

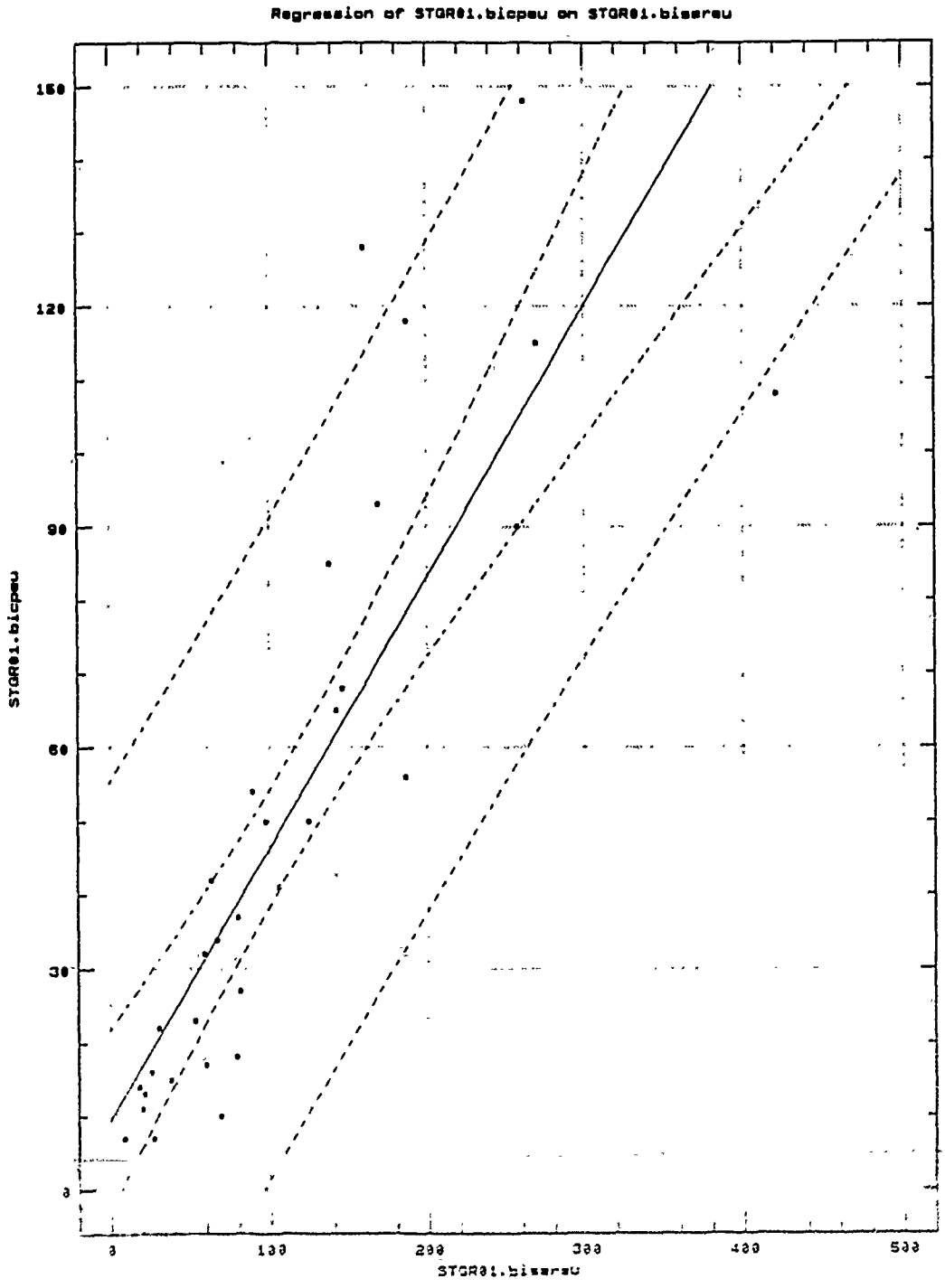

Regression of STQR01.bicpeu on STQR01.bisgrau
150
120
90
60
30
0
STQR01.bicpeu
0
100
200
300
400
500
STQR01.bisgrau

FIGURE 7.

Regression analysis of the relationship between serum and capillary blood antibody levels to *all microorganisms*. The X-axis denotes serum antibody levels to bacteria and the Y-axis denotes capillary blood antibody levels to the bacteria. The points represent individual patient levels with a group size of 96. The solid line represents a linear regression line. The inner dashed lines represent a 95% confidence interval and the outer dashed lines indicate the 99% confidence level for the regression.

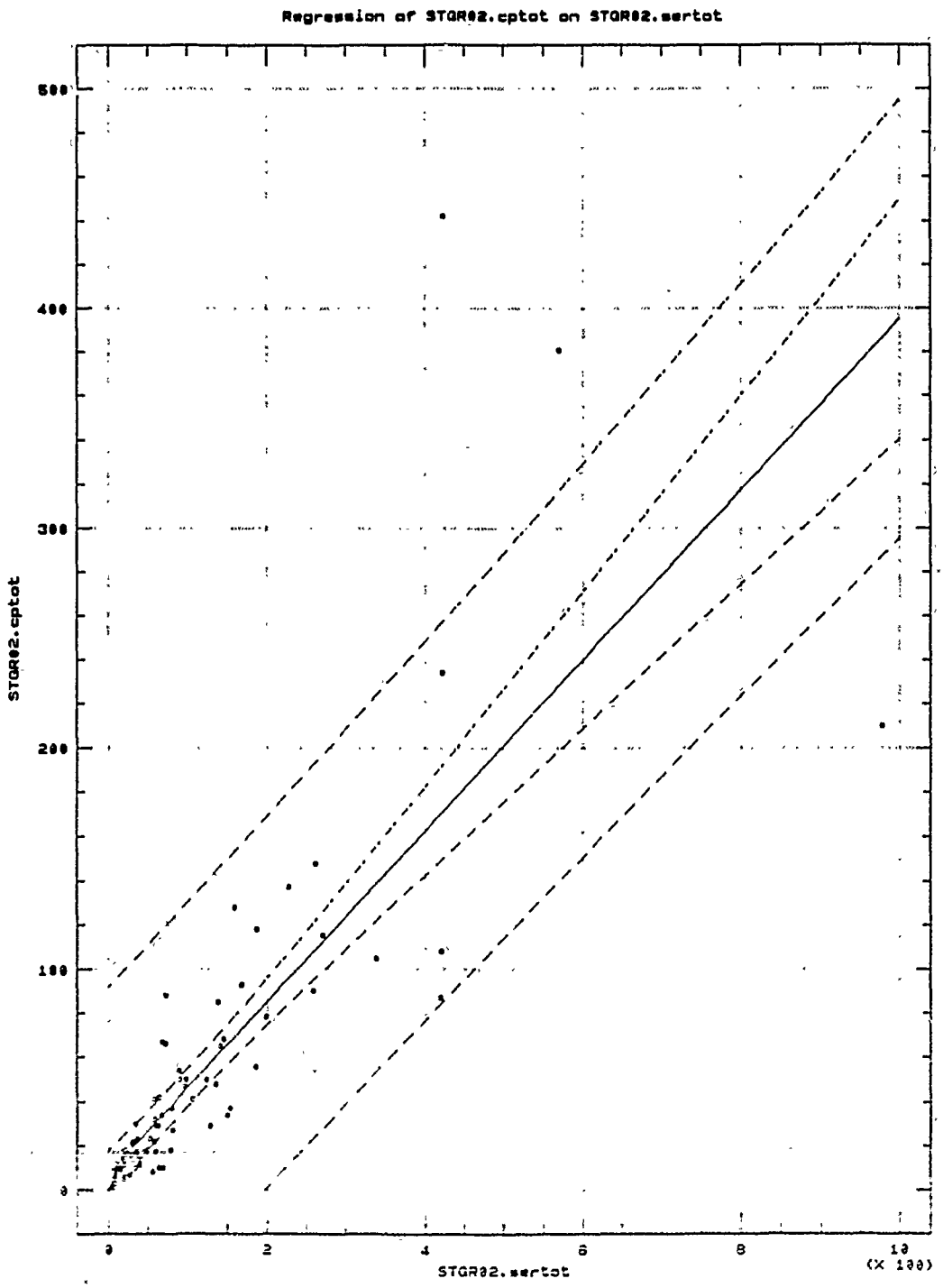

Regression of STGR02.cptot on STGR02.sertot
500
400
300
200
100
0
STGR02.cptot
0
2
4
6
8
10
(X 100)
STGR02.sertot

TABLE 4

SPEARMAN RANK CORRELATIONS (R_S)

	Aa	*Bξ*	*Bi*	**All Organisms**
N	*32*	*32*	*32*	*96*
R_S	*0.92*	*0.95*	*0.92*	*0.94*
p<	*0.0001*	*0.0001*	*0.0001*	*0.0001*

Spearman Rank Correlation (r_S) between serum and capillary blood antibody levels as determined by ELISA

TABLE 4

SPEARMAN RANK CORRELATIONS (R_S)

	Aa	*Bg*	*Bi*	**All Organisms**
N	*32*	*32*	*32*	*96*
R_S	*0.92*	*0.95*	*0.92*	*0.94*
p<	*0.0001*	*0.0001*	*0.0001*	*0.0001*

Spearman Rank Correlation (r_S) between serum and capillary blood antibody levels as determined by ELISA

distinction between elevated and normal antibody reactivities. The best concentration of serum would be that which produced a distinct colored "dot" when antibody titers to a given organism were elevated, and exhibited minimal or no color change when titers were normal.

Using various dilutions of antigen, the dot-blot assay was run with serum dilutions of 1:10, 1:20, and 1:50. Filter paper strips containing serum with known elevation in antibody to each organism were placed over the antigen "dots". Strips with low reactivity to the same organism were then placed over antigen "dots" immediately adjacent. After incubation and development, the difference in color change between the various dilutions of high and low reactive sera were assessed (Plate 1). For all organisms, the more dilute the serum, the more distinct the difference in color. Thus, a distinction between positive and negative responses was more easily made with a 1:50 dilution than with a 1:10 or 1:20 dilution. However, dilution beyond 1:50 resulted in the need for higher antigen concentrations. Therefore, a serum dilution of 1:50 was chosen.

During these experiments, it was noted that antigen concentration affected the color difference to a greater extent than serum dilution. In fact, minor alteration in serum dilution generally had little effect on the distinction between positive and negative reactions.

3. Selection of Appropriate Antigen Dilution.

Once a serum dilution of 1:50 was selected, the assay was performed using multiple concentrations of each antigen. Six sera with known high or low reactivity were selected for each organism. Antigen concentrations varied from 1×10^5 to 1×10^9 organisms per 10 µl drop (Plate 2). With each successive assay, the concentration range for the organisms was narrowed. For *B.gingivalis*, a concentration of 1×10^8 organisms per 10 µl allowed positive identification of 6/6 elevated sera; however, 1/6 negative sera were falsely identified as positive, and 2 others were considered "borderline elevated". A concentration of 1×10^7 gave

PLATE 1.

Variation in serum dilution (1:10, 1:20, 1:50) with multiple concentrations of *Aa*. Last column is background (BKG).

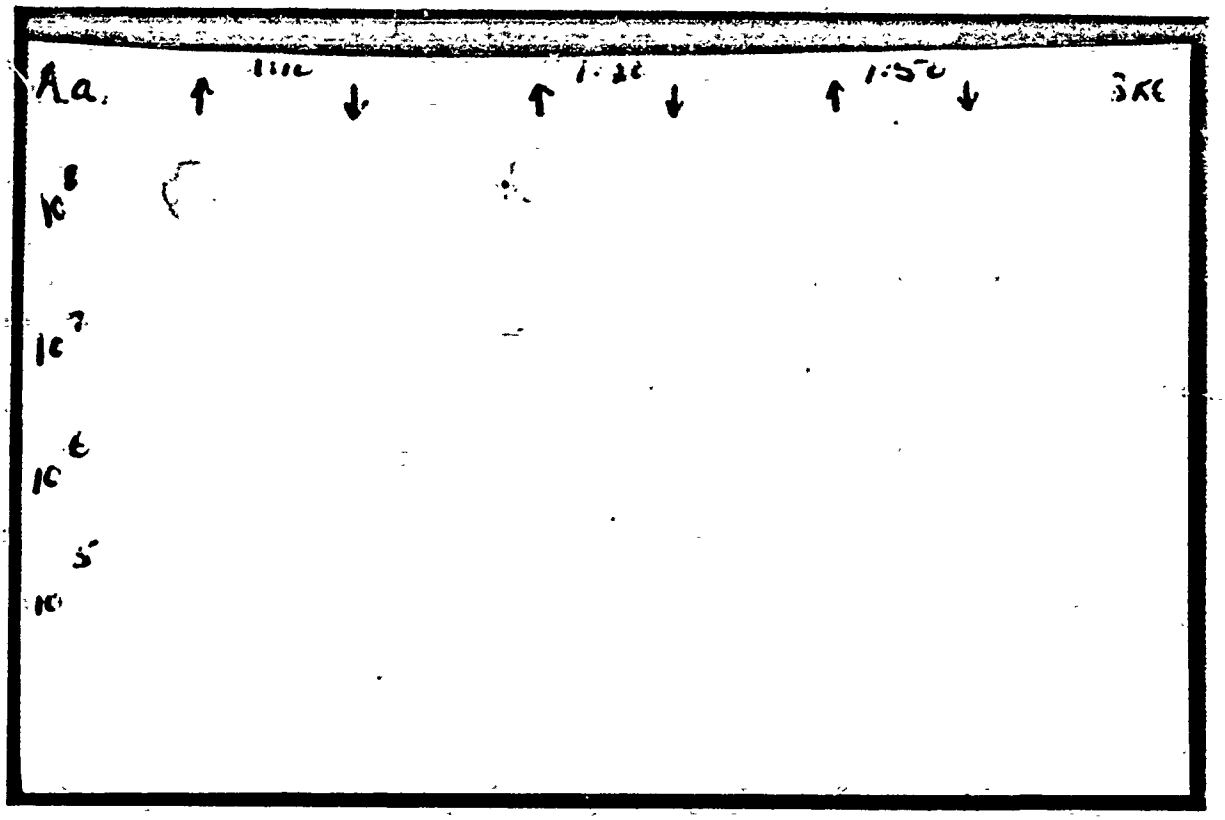
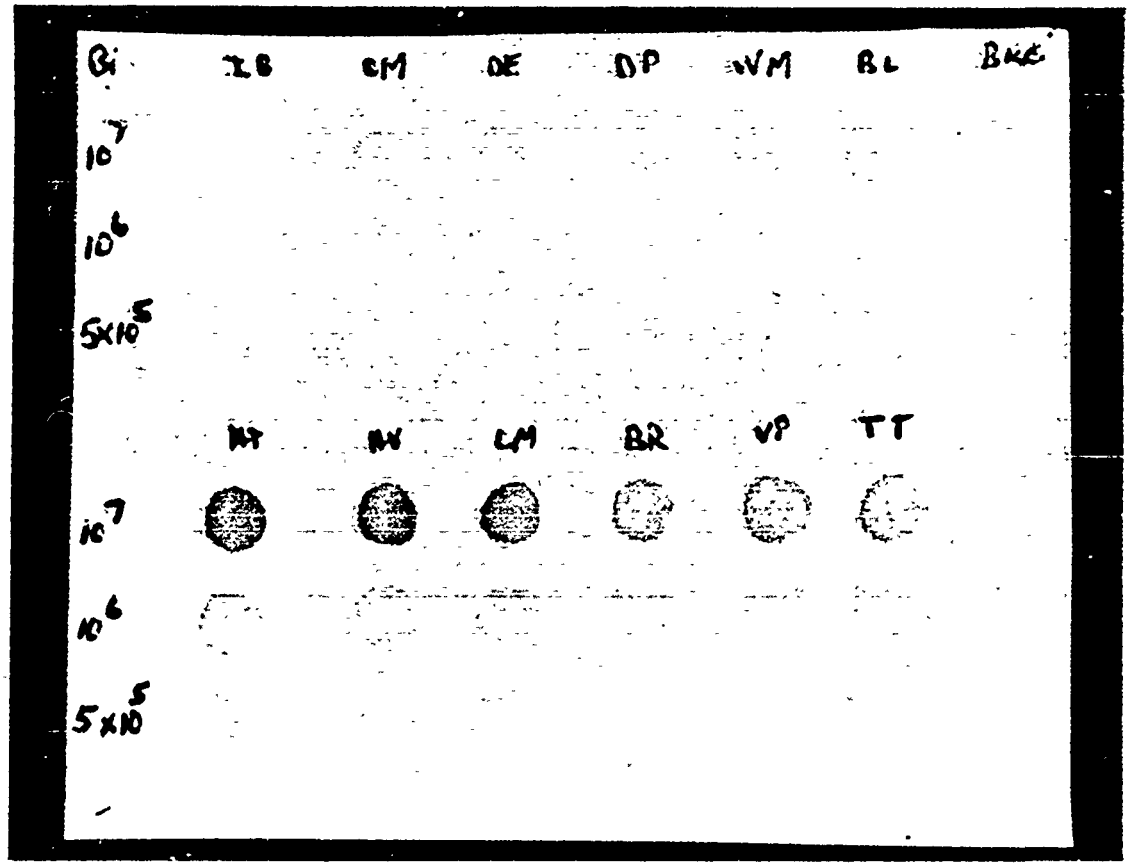

Aa.
1.1c
1.3c
1.5c
3KC
10^8
10^7
10^6
10^5

PLATE 2.

Variation in antigen concentration for *Bi*. Top six experimental samples are low reactive sera, bottom six are high reactive sera. Concentration of 1×10^6 organisms gives best distinction between elevated and normal sera.


Bi
LB
CM
DE
DP
VM
BL
BKE
10^7
10^6
5×10^5
MT
MV
CM
BR
VP
TT
10^7
10^6
5×10^5

positive identification of 6/6 elevated sera and negative identification of 6/6 normal sera. Thus, for *B.gingivalis*, a final concentration of 1×10^7 organisms/10 µl was chosen.

For *B.intermedius*, 1×10^7 organisms correctly identified 6/6 elevated sera, but produced false positives in 5/6 normal sera. 5×10^5 organisms/10 µl presented no false positives, but correctly identified only 4/6 elevated sera. A concentration of 1×10^6 *B.intermedius* per 10 µl positively identified 6/6 elevated sera with no false positives and was therefore selected as the concentration of choice.

A concentration of 1×10^8 *A.actinomycetemcomitans* per 10 µl drop enabled positive identification of 6/6 elevated sera, but also identified 2/6 normal sera as positive. 5×10^7 organisms reduced the false positive rate to 1/6 with no adverse effect on positive verification of truly elevated sera. Reduction of the concentration to 1×10^7 resulted in elimination of false positives, but reduced positive identification of elevated sera to 3/6. A concentration of 2.5×10^7 per 10 µl drop was therefore selected for *A.actinomycetemcomitans*.

4. Selection of Blocking Concentration.

Powdered non-fat milk was used to block non-specific binding sites after antigens were "spotted" onto the nitrocellulose sheets. Concentration of blocking agent was varied from 1% to 5% in TBS, while blocking time ranged from 30 minutes to 2 hours. With each variation in time and concentration, the degree of non-specific color change outside the antigen "dots" and within the area covered by the serum-containing strips was assessed. Because many of the color changes in the dot-blot assay are quite subtle, excellent blocking of non-specific binding sites is necessary. In general, the higher the concentration of powdered milk, the less time was required for adequate blocking. With 1% milk, adequate blocking was not achieved in under 90 minutes. When the concentration was increased to 5%, adequate blocking was achieved in 30 minutes. Due to the importance of this step in the ability to "read" the results, it was decided to block for 60 minutes with 5% powdered milk. In addition, prolonging the blocking time does not alter the time

needed to run the assay since all steps up to and including blocking are accomplished before samples are taken from the subjects. As mentioned, the nitrocellulose can be dried and stored for future use after antigen incubation or after blocking.

5. Selection of Incubation Time for Antibody-Containing Fluid.

Filter paper strips were cut in sections approximately 1 x 4 cm in size in order to completely cover the three antigen "dots" for each subject. Serum was diluted to 1:50 in TBS. Serum was placed in 15 ml covered test tubes and the filter strips were added to the tubes. The tubes were shaken and laid on their side to enable moistening of the strips. It was determined that 300 µl of serum solution was adequate to completely wet the filter strips. The serum-containing strips were then removed from the test tubes with a pair of tweezers and laid directly onto the antigen "dots". Incubation times were varied from 30 minutes to 2 hours. At 30 minutes, a number of elevated sera had produced only minor color change. By one hour, color change was more distinct and could be more readily distinguished from normal sera. Increasing incubation time to 2 hours offered no added benefit. Therefore, one hour was selected as an adequate time for incubation of antibody-containing fluid.

6. Selection of Enzyme Conjugate Reaction Time.

After incubation, the antibody-containing strips were removed from the nitrocellulose, which was then washed thoroughly with TBS and placed in a dish containing peroxidase-conjugated anti-human IgG. Concentrations of enzyme conjugate were varied from 1:1000 to 1:3000, while the reaction time ranged from 30-120 minutes. Again, in general, as concentration increased, reaction time decreased. At 1:3000, a reaction time of 60 or more minutes was needed to produce color changes equal to a 1:1500 concentration for 30 minutes. Increasing the concentration to 1:1000 offered no benefit relative to reaction time or color clarity. Therefore, a final enzyme conjugate concentration of 1:1500 and a reaction time of 30 minutes were selected.

7. Selection of Substrate Reaction Time.

The substrate for developing the peroxidase enzyme conjugate was made by combining 20 ml of methanol and 60 mg of 4-chloro-1-naphthol in one container and 100 ml of TBS plus 0.6 ml of 3% hydrogen peroxide in another container. When the nitrocellulose was ready for developing, the two solutions were mixed and the nitrocellulose added to the mixture. The reaction time for the substrate was critical for successful interpretation of results. Over-developing created non-specific color change of the entire nitrocellulose sheet. This greatly decreased the ability to assess subtle color differences between experimental and control samples. Conversely, under-developing resulted in underestimation of degrees of color change. Using the ideal concentrations and reaction times of the various reagents previously discussed, the substrate reaction time was altered from 1-5 minutes. Reaction times less than 2 minutes resulted in substantial underestimation of color change, producing high numbers of false negatives (sera with elevated ELISA antibody titers but minimal color change on the nitrocellulose). However, reaction times of three minutes or greater resulted in heavy non-specific staining of the nitrocellulose. This made distinction of weakly positive "dots" difficult if not impossible. Thus, a substrate reaction time of exactly 2 minutes was chosen.

The reaction was stopped by placing the nitrocellulose in distilled water for one minute. The results were then read. In order to maintain the stability of the reaction and prevent non-specific color change from the substrate, the nitrocellulose was soaked in distilled water for 15 minutes and was hung to dry. The results were then verified when the sheet had dried.

B. Initial Results of Dot-blot Assay

Thirty-nine sera were selected from a pool of samples used in other studies in this laboratory. These sera were chosen because previous ELISA had shown them to possess elevated antibody titers to one or more of the organisms *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius*. The primary investigator was unaware of the ELISA titers for each subject. To maintain the blind nature of the

study, each subject was given a number for identification.

ELISA was re-accomplished to determine serum titers to the organisms listed. The dot-blot assay was then performed as described. Nitrocellulose sheets were arranged so that a column of three antigen "dots" were placed directly under the number for each subject. After blocking, a single serum-containing filter strip for each subject was placed over the three "dots" and incubated for one hour. Each nitrocellulose sheet had five to ten experimental sera plus a positive and negative control serum sample. Thus, each sheet had its own controls for direct comparison with experimental samples. Following a 30 minute incubation with peroxidase-conjugated anti-human IgG and two minute development with substrate, each antigen "dot" was graded as either positive or negative relative to control sera (Plate 3).

When an antigen "dot" possessed a visual color change with an intensity equal to or greater than the positive control "dot" on the same nitrocellulose sheet, the result was deemed a positive reaction. When the "dot" had a color intensity equal to or less than the negative control, the result was considered negative. Each "dot" was placed into one of these two categories, positive or negative. It became apparent in using the assay that color changes with intensity lying between the positive and negative controls sometimes occurred. Since the assay records visual results, these "dots" were compared closely to the positive and negative controls and then subjectively placed in the positive or negative category. In general, "dots" with color even slightly more intense than the negative control were graded as positive.

Qualitative results of the dot-blot assay were then compared to quantitative ELISA results for each subject and each organism (Table 5). Previous research has established mean antibody reactivity to *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius* in healthy and periodontally diseased populations (Ebersole *et al.* 1982a, Ebersole *et al.* 1986). Significantly elevated antibody titers were those greater than two standard deviations above the mean of the periodontally normal group. As such, a "cutoff point" to delineate elevated from non-elevated titers was determined for each organism. For *A.actinomycetemcomitans*, antibody titers above 100 EU were considered to be elevated. Similar values for *B.gingivalis* and *B.intermedius* were 20

PLATE 3.

Representation of inital use of dot-blot assay for assessing antibody reactivity in experimental serum samples. Relative to control sera, samples #32 and 33 have elevated reactivity to *Aa*, while samples #31, 32, and 33 are positive to *Bg*. Samples #31, 33, and 34 are positive to *Bi*.

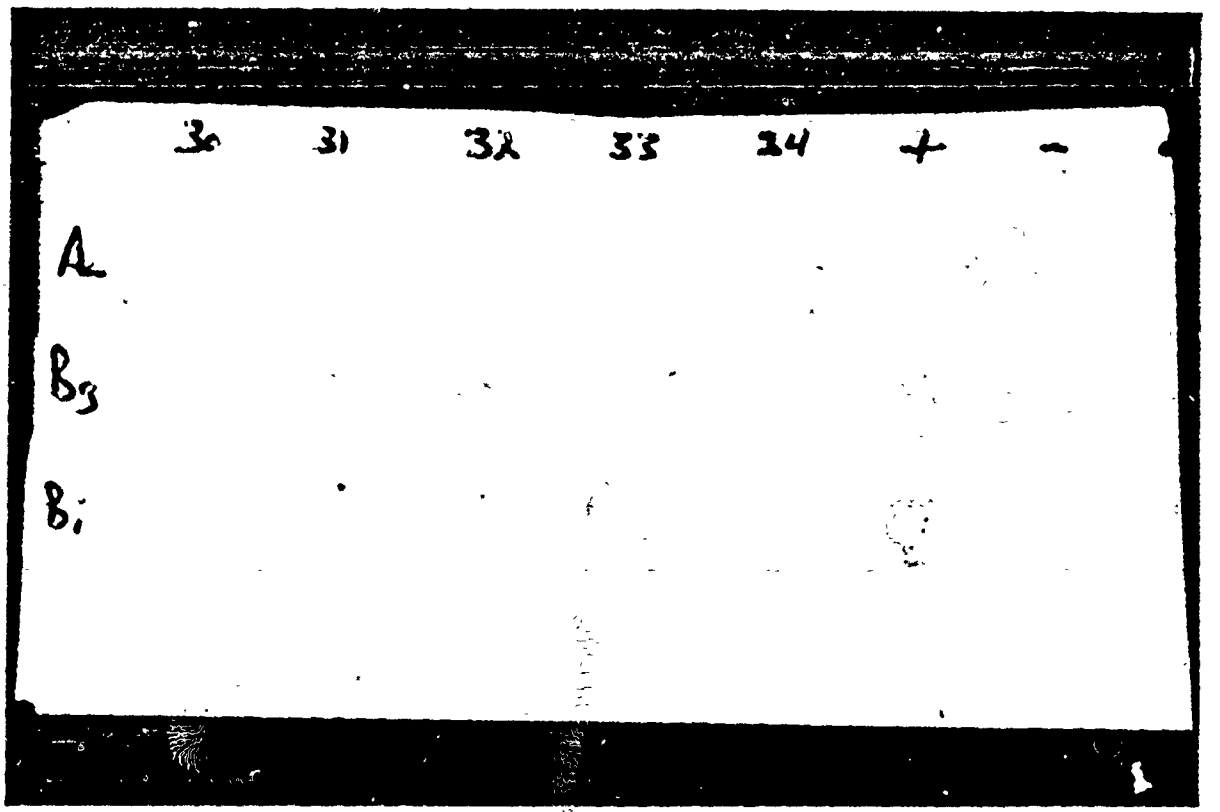

30
31
32
33
34
+
-
A
B3
B1

TABLE 5

DOT-BLOT ASSAY vs. ELISA for 39 SERUM SAMPLES

	Aa		*Bg*		*Bi*	
Pt No.	ELISA	DB	ELISA	DB	ELISA	DB
1	108	+	41	+	1070	+
2	221	-	4	-	41	-
3	27	-	15	+	143	+
4	41	-	40	+	114	+
5	64	-	154	+	126	+
6	14	-	12	+	35	-
7	55	-	47	+	62	-
8	178	+	13	+	282	+
9	570	+	26	-	191	+
10	34	-	30	+	96	-
11	15	-	5	-	15	-
12	69	+	32	+	23	-
13	102	+	203	+	145	+
14	129	+	53	+	21	-
15	41	+	215	+	21	+
16	272	+	16	+	26	-
17	484	+	8	-	110	+
18	23	+	12	+	21	-
19	22	-	17	+	88	+
20	15	-	21	+	48	+
21	31	-	50	+	58	-
22	28	-	21	+	38	+
23	45	-	61	+	317	+
24	73	-	15	-	203	-
25	218	+	32	+	53	-
26	126	+	18	+	29	-
27	268	+	8	-	29	-
28	39	-	33	+	9	-
29	269	+	0	-	129	+
30	88	+	339	+	239	+
31	726	+	83	+	26	-
32	304	-	1	-	66	-
33	179	-	0	-	108	-
34	76	+	797	+	158	+
35	215	+	322	+	158	+
36	91	-	1	-	49	+
37	120	+	2	-	102	+
38	556	-	2	+	14	-
39	126	+	0	-	33	+

Note: ELISA titers which delineate elevated response from normal response are:
Aa = 100 EU
Bg = 20 EU
Bi = 125 EU

and 125 EU respectively. In this way, quantitative ELISA data could be categorized for each subject and each organism as either elevated or non-elevated based on the "cutoff point". These data could then be compared to the qualitative dot-blot data. Thus, each "dot" on the nitrocellulose sheet was graded as positive or negative and was then compared to the ELISA data for that particular organism and subject.

The sensitivity and specificity of the dot-blot assay was determined for each organism and for all organisms combined using ELISA as the "gold standard" (Table 6). In this regard, sensitivity was defined as the percentage of elevated sera (as determined by ELISA) which demonstrated positive reactions in the dot-blot assay. Specificity was the percentage of non-elevated sera (by ELISA) which tested negative in the dot-blot. False positive values were those which were deemed positive by dot-blot, but were negative by ELISA. Conversely, false negative reactions were those in which sera with elevated antibody reactivity by ELISA were deemed negative by dot-blot (Table 7).

For the 39 serum samples tested, the sensitivity of the dot-blot relative to the ELISA ranged from 0.79 to 0.95 for each individual organism and was 0.88 (45/51) when reactivity to all organisms was combined. Specificity values for individual bacteria ranged from 0.58 to 0.75, with an overall specificity of 0.67 (44/66). The total number of false negatives was quite low (6/51 or 11.8%), indicating that the dot-blot assay rarely showed a negative reaction for sera with elevated antibody titers. The number of false positives was considerably higher (22/66 or 33%), signifying a greater chance of identifying sera with normal antibody reactivity by ELISA as positive by the dot-blot assay.

C. Adjusted Capillary Antibody Reactivity

In order to create a simple and rapid assay, the use of capillary blood was preferred over serum. To elucidate the effect that variation in antibody reactivity between capillary blood and serum might have on the use of the dot-blot assay, adjusted capillary titers were calculated in two ways (Table 8). First, the mean serum

TABLE 6

CONTINGENCY TABLES for 39 SERUM SAMPLES

Aa (≥ 100 EU)

		ELISA +	ELISA -
DB	+	15	5
	-	4	15

Sens = 0.79
Spec = 0.75

Bg (≥ 20 EU)

		ELISA +	ELISA -
DB	+	19	8
	-	1	11

Sens = 0.95
Spec = 0.58

Bi (≥ 125 EU)

		ELISA +	ELISA -
DB	+	11	9
	-	1	18

Sens = 0.92
Spec = 0.67

Total sens/spec

		ELISA +	ELISA -
DB	+	45	22
	-	6	44

Sens = 0.88
Spec = 0.67

TABLE 7

DETERMINATION of SENSITIVITY, SPECIFICITY, POSITIVE PREDICTIVE VALUE and NEGATIVE PREDICTIVE VALUE

ELISA

DB		+	-
	+	*a*	*b*
	-	*c*	*d*

OR

PERIODONTAL DISEASE

DB		+	-
	+	*a*	*b*
	-	*c*	*d*

Sens = a/(a+c)
Spec = d/(b+d)
False pos = b
False neg = c
Pv(+) = a/(a+b)
Pv(-) = d/(c+d)

TABLE 8

ADJUSTED CpEU

	Aa	*Bg*	*Bi*	All Organisms
N	*32*	*32*	*32*	*96*
SerEU	*150.5*	*19.3*	*111.4*	*93.7*
CpEU	*68.7*	*14.4*	*50.4*	*44.5*
% Cp	*47.0*	*68.0*	*48.8*	
% CpTot	*54.6*	*54.6*	*54.6*	*54.6*
AdjCp	*70.7*	*13.1*	*54.4*	
AdjCpTot	*82.2*	*10.5*	*60.8*	*51.2*

SerEU = actual serum EU by ELISA

CpEU = actual capillary EU by ELISA

%Cp = CpEU as % of SerEU for each individual organism

%CpTot = CpEU as % of SerEU for all organisms combined

AdjCp = SerEU x %Cp

AdjCpTot = SerEU x %CpTot

antibody titer to each bacterial species (SerEU) was multiplied by the percent capillary reactivity for each individual organism relative to the serum reactivity to that organism (%Cp) [AdjCp = SerEu x %Cp]. For example, for *A.actinomycetemcomitans*, the mean serum antibody titer was 150.5 EU (SerEU = 150.5). For this same organism, capillary antibody reactivity was found to be approximately 47% that of serum (%Cp = 0.47). Therefore, the adjusted capillary titer (AdjCp) for *A.actinomycetemcomitans* was calculated by the equation [SerEU x %Cp= AdjCp] or 150.5 x 0.47 = 70.7 EU. The second method for calculating adjusted capillary titers involved multiplying the SerEu and the percent capillary reactivity to all organisms relative to the serum reactivity to all organisms (%CpTot) [AdjCpTot = SerEu x %CpTot]. When serum and capillary reactivities to all organisms were compared, it was determined that the capillary titer was 54.6% that of serum (%CpTot = 0.546). Thus, for *A.actinomycetemcomitans*, AdjCpTot = 150.5 x 0.546; AdjCpTot = 82.2 EU.

Adjusted capillary titers (AdjCp and AdjCpTot) were then compared to the capillary reactivity measured directly by ELISA (CpEU). Adjusted capillary titers (AdjCp) based on the percent capillary reactivity relative to serum activity for each individual organism (%Cp) were very similar to measured capillary ELISA titers (CpEU), with differences of $\pm$ 1.3-4.0 EU (2.9-9.0%). For example, the mean capillary reactivity to *A.actinomycetemcomitans* as determined by ELISA was 68.7 EU (CpEU = 68.7). This compares favorably to the AdjCP of 70.7 for this organism. When capillary titers (AdjCpTot) were adjusted by the percent capillary reactivity relative to serum activity for all organisms combined (%CpTot), the differences were somewhat greater ($\pm$ 3.9-13.5 EU or 19.7-27.1%). For *A.actinomycetemcomitans*, the AdjCpTot was 82.2 EU compared to the ELISA capillary titer (CpEU) of 68.7 EU.

The relationships between the adjusted capillary antibody titers (AdjCp and AdjCpTot) and the titers measured by ELISA (CpEU) were analyzed by the Wilcoxon Paired Ranks Test (Table 9). There were no statistically significant differences in values adjusted by %Cp or %CpTot and those values derived from the ELISA. That is, AdjCp and AdjCpTot values were not significantly different from CpEU values.

The dot-blot assay was designed such that only a single dilution of capillary

TABLE 9

WILCOXON PAIRED RANKS TEST
COMPARISON of PAIRED VALUES

	Aa	*Bg*	*Bi*	All Organisms
N (pairs)	*32*	*32*	*32*	*96*
CpEU:AdjCp p<	*0.649*	*0.743*	*0.715*	
CpEU:AdjCpTot p<	*0.339*	*0.783*	*0.415*	*0.494*

No statistical differences between capillary antibody titers as measured by ELISA and capillary reactivities adjusted by %Cp and %CpTot

blood would be needed. This allowed a single blood-containing strip to be placed over all three antigen "dots". While the assay utilizes experimental capillary blood, the positive and negative controls are sera. It has been demonstrated that capillary antibody reactivity is, on average, only 54.6% (%CpTot) that of serum at the same dilution. Therefore, use of identical dilutions of serum and capillary blood in the assay would result in underestimation of capillary reactivity since a direct comparison is made between experimental capillary blood and control sera on the same nitrocellulose sheet. Thus, it was necessary to alter the dilution of either the serum or the capillary blood.

As shown in Tables 8 and 9, adjustment of the capillary antibody titers to account for the lower reactivity in capillary blood resulted in values not significantly different from values measured by ELISA. Previously, a 1:50 serum dilution had been shown to provide the most accurate and readable results in the dot-blot assay. Thus, a 1:50 dilution of positive and negative control sera was chosen.

Based on the overall percent reactivity in capillary blood versus serum (%CpTot = 54.6%), a capillary dilution of 1:27 would have provided approximately the same antibody titer as a 1:50 serum dilution. As noted previously, the serum dilution did not appear to be the critical factor influencing the accuracy of the assay; rather, the concentration of antigen was primarily responsible for the accurate identification and differentiation of positive and negative results. In order to simplify the procedure, it was decided to utilize a capillary blood dilution of 1:25. Presumably, this would provide roughly equivalent antibody levels in serum and capillary blood samples from any given patient.

In order to assess the similarity between serum antibody titers at 1:50 dilution and capillary titers at 1:25 dilution, the mean SerEU values were divided by a factor of 2 to produce adjusted capillary EU values (AdjCpEU). The AdjCpEU figures were then compared to the actual capillary titers (CpEU). Results are presented in Table 10. While the AdjCpEU values were higher than CpEU for *A.actinomycetemcomitans* and *B.intermedius*, the opposite occurred for *B.gingivalis*. Overall, when antibody titers to

TABLE 10

USE of EXPERIMENTAL CAPILLARY BLOOD at 1:25 DILUTION vs. CONTROL SERA at 1:50 DILUTION

	Aa	*Bg*	*Bi*	All Organisms
SerEU	*150.5*	*19.3*	*111.4*	*93.7*
CpEU	*68.7*	*14.4*	*50.4*	*44.5*
AdjCpEU	*75.3*	*9.7*	*55.7*	*46.9*

AdjCpEU derived by dividing SerEU by 2

all organisms were combined, the CpEU and AdjCpEU values were quite similar (44.5 and 46.8 respectively). Thus, doubling the dilution factor for the control sera to 1:50 would theoretically provide similar titers to capillary blood at a dilution of 1:25.

D. Use of Dot-blot Assay in Screening of Periodontal Patients

The dot-blot assay and ELISA were used to evaluate 34 patients with various forms of periodontal disease. A 1:25 dilution of capillary blood and a 1:50 dilution of control sera were used. Color changes on the nitrocellulose sheets were read in a blind fashion by an investigator unaware of the ELISA results (Plates 4 & 5). Qualitative dot-blot results were then compared to quantitative ELISA data for each organism and each subject (Table 11). The ability of the dot-blot assay to distinguish positive from negative responders was assessed. Contingency tables were designed to analyze the sensitivity and specificity of the dot-blot assay relative to the ELISA "gold standard" (Table 12).

Sensitivity values (Table 12) were 0.83 for *A.actinomycetemcomitans* and 1.00 for *B.gingivalis* and *B.intermedius*. That is, 100% of subjects with elevated ELISA titers to the *Bacteroides* species and 83% of those with elevated antibody to *A.actinomycetemcomitans* demonstrated a positive response to these organisms by dot-blot. Specificity values for *A.actinomycetemcomitans* and *B.gingivalis* were 0.82, while the dot-blot showed a specificity of 0.86 for antibody to *B.intermedius*. Thus, 82% and 86% of subjects with normal ELISA titers to these organisms displayed negative dot-blot responses.

Data for responses to all organisms in all subjects were then grouped into a single contingency table (Table 12; last contingency table). From a total of 37 elevated ELISA titers, 35 were positive by the dot-blot assay (sensitivity = 0.95). Likewise, of 65 normal ELISA titers, 54 were negative by dot-blot (specificity = 0.83).

Contingency tables for each individual organism were analyzed using Fisher's Exact Test to determine the significance of the relationship between dot-blot and ELISA values (Table 12). Levels of significance were: *A.actinomycetemcomitans* $p=0.00036$, *B.gingivalis* $p<0.00001$, and *B.intermedius* $p<0.00001$. The contingency table for data from all organisms combined was examined by Chi-square analysis, with

PLATE 4.

Use of dot-blot assay for periodontally diseased subjects. Whole capillary blood at a 1:25 dilution is compared to control sera at a 1:50 dilution. Relative to positive and negative controls, subject #2 has positive responses to *Bg* and *Bi*, while subject #3 is positive to *Aa* and *Bg*. The response to *Aa* for subject #2 was considered "weakly" positive relative to the positive and negative controls for *Aa*.

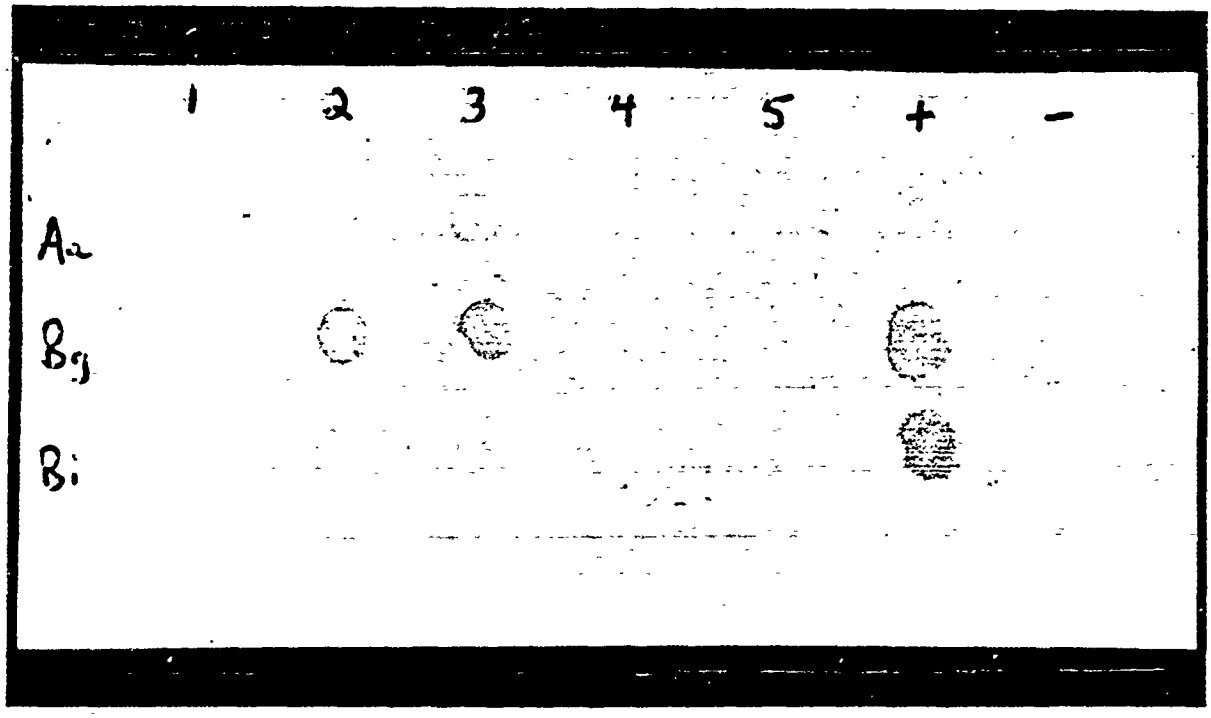

1
2
3
4
5
+
-
A2
Bg
Bi

PLATE 5.

Use of dot-blot assay for periodontally diseased subjects. Whole capillary blood at a 1:25 dilution is compared to control sera at a 1:50 dilution. Relative to positive and negative controls, subject #22 has positive responses to all three organisms, while subject #23 is positive to *Bi*. Subject #24 has elevated responses to *Bg* and *Bi*.

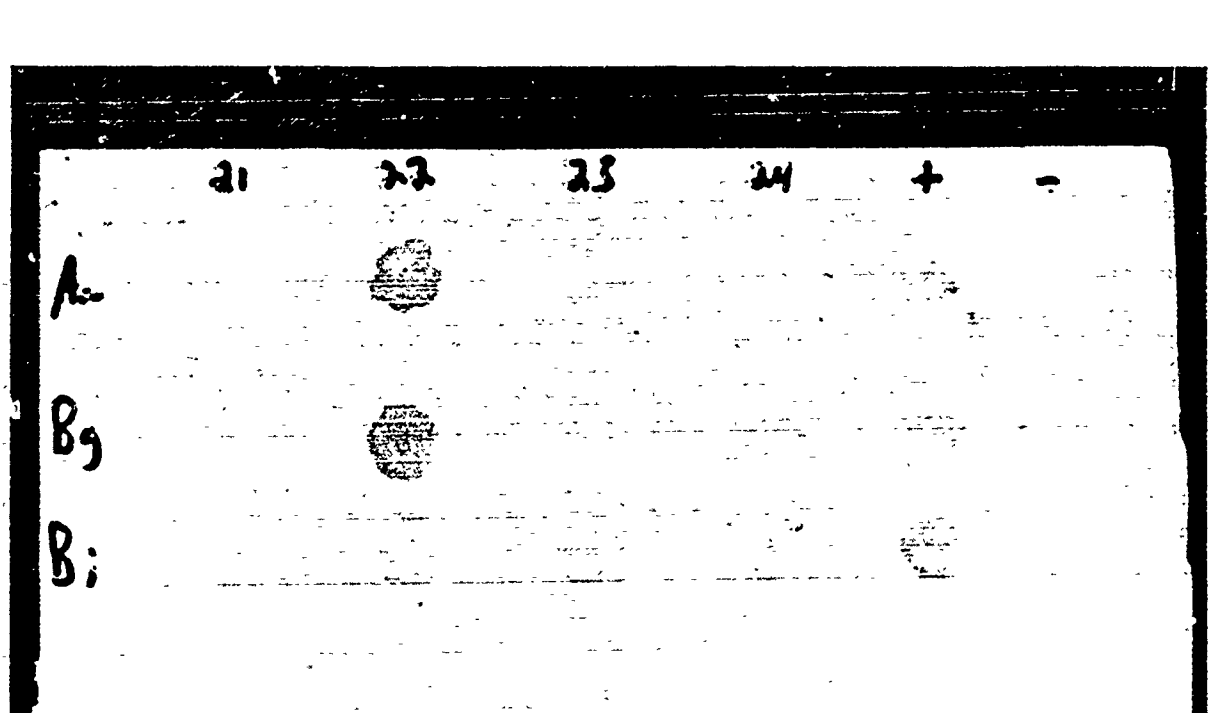

21
22
23
24
+
-
A
Bg
Bi

TABLE 11

DOT-BLOT ASSAY vs. ELISA for 34 PERIODONTALLY DISEASED SUBJECTS

	Aa		*Bg*		*Bi*	
Pt No.	ELISA	DB	ELISA	DB	ELISA	DB
1	339	+	17	-	67	-
2	7	-	30	+	262	+
3	56	-	45	+	53	+
4	129	+	13	-	421	+
5	16	-	8	-	81	-
6	22	+	12	+	146	+
7	15	-	40	+	64	+
8	154	-	8	+	26	-
9	420	+	18	+	258	+
10	57	+	7	-	31	-
11	422	+	5	-	125	+
12	9	-	3	-	38	-
13	28	-	3	-	60	-
14	150	+	68	+	142	+
15	35	-	35	+	18	-
16	89	+	7	+	142	+
17	48	-	3	-	86	-
18	570	+	2	-	90	+
19	22	-	73	+	59	-
20	13	-	1	-	20	-
21	63	-	4	-	21	-
22	228	+	37	+	160	+
23	61	-	3	-	80	-
24	30	-	21	+	98	-
25	64	-	3	-	69	-
26	12	-	1	-	186	+
27	423	+	36	+	138	+
28	199	-	2	-	9	-
29	91	+	8	-	169	+
30	59	-	2	-	27	-
31	96	-	49	+	187	+
32	978	+	73	+	79	-
33	136	+	20	+	270	+
34	49	-	0	-	106	-

Note: ELISA titers which delineate elevated response from normal response are: *Aa* = 100 EU
Bg = 20 EU
Bi = 125 EU

TABLE 12

CONTINGENCY TABLES for 34 PERIODONTALLY DISEASED SUBJECTS

Aa (≥ 100 EU)

		ELISA +	ELISA -
DB	+	10	4
	-	2	18

Sens = 0.83
Spec = 0.82
p = 0.00036

Bg (≥ 20 EU)

		ELISA +	ELISA -
DB	+	12	4
	-	0	18

Sens = 1.00
Spec = 0.82
p< 0.00001

Bi (≥ 125 EU)

		ELISA +	ELISA -
DB	+	13	3
	-	0	18

Sens = 1.00
Spec = 0.86
p< 0.00001

Total sens/spec

		ELISA +	ELISA -
DB	+	35	11
	-	2	54

Sens = 0.95
Spec = 0.83
p< 0.000001

Fisher's Exact Test for *Aa*, *Bg*, *Bi*
Chi-square analysis for Total sens/spec

$p < 0.000001$. Thus, the results from the dot-blot assay and those from the ELISA demonstrated a highly statistically significant relationship for capillary blood. These data demonstrate that the dot-blot assay very closely reflected the systemic antibody reactivity of the subjects as determined by ELISA.

E. Screening of Periodontally Healthy Subjects

Serum and capillary blood samples were obtained from ten periodontally healthy subjects. Serum antibody titers to the three organisms were determined by ELISA. Capillary blood samples were then analyzed by dot-blot assay. Results were compared as described above for each subject (Table 13). For this group of periodontally normal patients, the dot-blot assay displayed an overall sensitivity of 0.80 and a specificity of 0.96 (Table 14). Surprisingly, 3/10 healthy subjects had elevated antibody to *B.gingivalis* by both ELISA and dot-blot.

F. Use of ELISA to Distinguish Periodontally Healthy and Diseased Subjects

The literature is replete with studies demonstrating the prevalence of increased antibody to putative periodontopathogens in patients with periodontal disease. The dot-blot assay and the ELISA were examined for their ability to correctly identify periodontally healthy and diseased patients (Table 15 and 16).

In this context, sensitivity is defined as the number of diseased subjects with a positive ELISA or dot-blot result divided by the total number of diseased subjects. Specificity is the number of periodontally healthy subjects with a negative test result divided by the total number of healthy subjects. The positive predictive value, or predictive value of a positive test, is the number of positive tests in diseased subjects divided by the total number of positive tests. The negative predictive value or predictive value of a negative test is defined as the number of negative tests in healthy subjects divided by the total number of negative tests.

An elevated ELISA titer to *B.intermedius* was predictive of a diseased status in 13/34 patients (sensitivity = 0.38), while elevated titers to *B.gingivalis* or *A.actinomycetemcomitans* were predictive of disease in 12/34 instances

TABLE 13

DOT-BLOT ASSAY vs. ELISA for 10 PERIODONTALLY HEALTHY SUBJECTS

	Aa		*Bg*		*Bi*	
Pt No.	ELISA	DB	ELISA	DB	ELISA	DB
1	47	-	3	-	33	-
2	95	-	36	+	137	-
3	81	+	41	+	19	-
4	33	-	2	-	52	-
5	56	-	2	-	34	-
6	67	-	3	-	134	+
7	75	-	4	-	46	-
8	65	-	41	+	37	-
9	45	-	4	-	50	-
10	17	-	9	-	21	-

Note: ELISA titers which delineate elevated response from normal response are: *Aa* = 100 EU
Bg = 20 EU
Bi = 125 EU

TABLE 14

CONTINGENCY TABLES for 10 PERIODONTALLY HEALTHY SUBJECTS

Aa (≥ 100 EU)

		ELISA +	ELISA -
DB	+	0	1
	-	0	9

Sens = none elevated by ELISA
Spec = 0.90

Bg (≥ 20 EU)

		ELISA +	ELISA -
DB	+	3	0
	-	0	7

Sens = 1.00
Spec = 1.00

Bi (≥ 125 EU)

		ELISA +	ELISA -
DB	+	1	0
	-	1	8

Sens = 0.50
Spec = 1.00

Total sens/spec

		ELISA +	ELISA -
DB	+	4	1
	-	1	24

Sens = 0.80
Spec = 0.96

TABLE 15

CONTINGENCY TABLES EXAMINING ABILITY of ELISA to DELINEATE PERIODONTAL STATUS

Aa (≥ 100 EU)

		PERIODONTAL DISEASE +	PERIODONTAL DISEASE -
DB	+	12	0
	-	22	10

Sens = 0.35
Spec = 1.00
Pv(+)= 1.00
Pv(-)= 0.31

Bg (≥ 20 EU)

		PERIODONTAL DISEASE +	PERIODONTAL DISEASE -
DB	+	12	3
	-	22	7

Sens = 0.35
Spec = 0.70
Pv(+)= 0.80
Pv(-)= 0.24

Bi (≥ 125 EU)

		PERIODONTAL DISEASE +	PERIODONTAL DISEASE -
DB	+	13	2
	-	21	8

Sens = 0.38
Spec = 0.80
Pv(+)= 0.87
Pv(-)= 0.28

Total sens/spec

		PERIODONTAL DISEASE +	PERIODONTAL DISEASE -
DB	+	23	4
	-	11	6

Sens = 0.68
Spec = 0.60
Pv(+)= 0.85
Pv(-)= 0.35

TABLE 16

CONTINGENCY TABLES EXAMINING ABILITY of DOT-BLOT ASSAY to DELINEATE PERIODONTAL STATUS

Aa (≥ 100 EU)

		PERIODONTAL DISEASE +	PERIODONTAL DISEASE -
DB	+	14	1
	-	20	9

Sens = 0.41
Spec = 0.90
Pv(+) = 0.93
Pv(-) = 0.31

Bg (≥ 20 EU)

		PERIODONTAL DISEASE +	PERIODONTAL DISEASE -
DB	+	16	3
	-	18	7

Sens = 0.47
Spec = 0.70
Pv(+) = 0.84
Pv(-) = 0.28

Bi (≥ 125 EU)

		PERIODONTAL DISEASE +	PERIODONTAL DISEASE -
DB	+	16	1
	-	18	9

Sens = 0.47
Spec = 0.90
Pv(+) = 0.94
Pv(-) = 0.33

Total sens/spec

		PERIODONTAL DISEASE +	PERIODONTAL DISEASE -
DB	+	23	4
	-	11	6

Sens = 0.68
Spec = 0.60
Pv(+) = 0.85
Pv(-) = 0.35

(sensitivity = 0.35). Thus, 35% of subjects with periodontal disease had elevated ELISA reactivity to *B.gingivalis* or *A.actinomycetemcomitans*, and 38% had elevated titers to *B.intermedius*. Conversely, a normal ELISA titer to *A.actinomycetemcomitans* was predictive of a periodontally healthy state in 10/10 subjects (specificity = 1.00). Specificity values for *B.intermedius* and *B.gingivalis* were 0.80 and 0.70 respectively. Therefore, 100% of periodontally healthy subjects had normal ELISA antibody titers to *A.actinomycetemcomitans*. Eighty percent of healthy patients had normal titers to *B.intermedius* and 70% had normal reactivity to *B.gingivalis*.

The positive predictive values for *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius* were 1.00, 0.80, and 0.87 respectively. Hence, 100% of subjects with elevated ELISA titers to *A.actinomycetemcomitans* had periodontal disease, 80% of those with elevated reactivity to *B.gingivalis* were diseased, and 87% of patients with elevated ELISA titers to *B.intermedius* had periodontal disease.

The negative predictive values ranged from 0.24-0.31. Thus, only 24-31% of patients with normal ELISA titers to *A.actinomycetemcomitans*, *B.gingivalis*, or *B.intermedius* were periodontally healthy. Stated another way, 76% of patients with normal ELISA titers to these organisms were actually periodontally diseased. The low negative predictive values are to be expected, since this study population was skewed toward a high prevalence of disease. In addition, many patients with periodontal disease have normal titers to one or more of these organisms. In contrast, the high positive predictive values for the ELISA indicate that subjects who demonstrated elevated titers to one of these organisms had a strong chance of having periodontal disease.

Finally, the overall ELISA response of each subject to the entire panel of three organisms was used as a predictor of periodontal health or disease (Table 15; last contingency table). Thus, an elevated ELISA titer to one or more of the organisms was used to predict periodontal disease while a negative response to all three organisms was used to predict a state of health. The overall sensitivity of the ELISA in this regard was 0.68 (23/34). Accordingly, 23 of 34 periodontally diseased patients demonstrated an elevated ELISA response to one or more of the three organisms tested. Overall

specificity was 0.60; i.e., 6 of the 10 periodontally healthy subjects had a negative response to all three organism by ELISA. The predictive value of a positive test was 0.85; hence, 23 of 27 subjects with an elevated ELISA to one or more organisms did indeed have periodontal disease. The predictive value of a negative test was 0.35; namely, only 6 of 17 subjects who demonstrated normal ELISA responses to all three organisms were periodontally healthy.

Consequently, subjects who had elevated ELISA antibody titers to one or more of the organisms had a high probability of also having some form of periodontal disease (high positive predictive value), while the mere absence of an elevated response did not necessarily indicate a state of periodontal health (low negative predictive value). Contingency tables were analyzed by Fisher's Exact Test and Chi-square analysis to assess the relationship between the presence or absence of elevated antibody titers to the organisms as determined by ELISA and the presence or absence of periodontal disease (Table 17). There was a statistically significant relationship between presence or absence of elevated titer to *A.actinomycetemcomitans* and presence or absence of disease ($p=0.026$). For *B.gingivalis* and *B.intermedius*, this relationship did not reach a level of statistical significance ($p=0.536$ and $p=0.250$ respectively). By Chi-square analysis, the overall ELISA response of the subjects to the entire panel of three organisms as an indicator of the presence or absence of disease did not reach significance ($p=0.114$).

G. Use of Dot-blot to Distinguish Periodontally Healthy and Diseased Subjects

Like the ELISA, the dot-blot assay was analyzed for its ability to predict a state of periodontal health or disease (Table 16). The sensitivity of the dot-blot assay in identifying a diseased state by a positive reaction to a single organism ranged from 0.41- 0.47. Thus, 41% of periodontally diseased subjects had a positive dot-blot response to *A.actinomycetemcomitans*, 47% were positive to *B.gingivalis*, and 47% were positive to *B.intermedius*. Specificity ranged from 0.70-0.90. Seventy percent of

periodontally healthy subjects had a negative dot-blot response to *B.gingivalis*, while 90% were negative to *B.intermedius* and *A.actinomycetemcomitans*.

The positive predictive value for *A.actinomycetemcomitans*, *B.gingivalis*, and B.intermedius were 0.93, 0.84, and 0.94 respectively. Consequently, 93% of subjects with a positive dot-blot response to *A.actinomycetemcomitans* had periodontal disease, while 84% of those with positive reactions to *B.gingivalis* and 94% with positive responses to *B.intermedius* were diseased. The negative predictive values ranged from 0.28-0.33.

When the overall dot-blot response to the panel of three organisms was used as a predictor of periodontal status, the dot-blot assay was found to have a sensitivity, specificity, positive predictive value, and negative predictive value identical to the ELISA (Table 16; last contingency table). Thus, the dot-blot assay and ELISA possessed equal capabilities in identifying periodontally diseased and healthy subjects using this panel of three organisms.

Contingency tables for the dot-blot assay were examined by Fisher's Exact Test and Chi-square analysis in an identical manner to analysis of ELISA responses (Table 17). The relationships between positive or negative dot-blot responses to *A.actinomycetemcomitans* or *B.gingivalis* and the presence or absence of periodontal disease were not statistically significant ($p=0.068$ and $p=0.279$ respectively). Positive or negative dot-blot responses to *B.intermedius* were significantly related to presence or absence of disease ($p=0.035$). By Chi-square analysis, the overall dot-blot response of the subjects to the panel of three organisms was not significantly related to the presence or absence of periodontal disease ($p=0.114$).

Thus, in this population of subjects, an elevated response to one or more of the organisms was indicative of disease in 85% of the individuals (positive predictive value =0.85). In addition, 68% of all periodontally diseased subjects were identified by a positive response to the test. A negative response to all organisms was indicative of periodontal health in only 35% of subjects (negative predictive value =0.35). These results are presented in the last contingency table displayed in Tables 15 and 16. Due

TABLE 17

STATISTICAL ANALYSIS of ABILITY of ELISA and DOT-BLOT ASSAY to DELINEATE PERIODONTAL STATUS

	Aa	*Bg*	*Bi*	All Organisms
N	*44*	*44*	*44*	*44*
ELISA p<	*0.026*	*0.536*	*0.250*	*0.114*
Dot-blot p<	*0.068*	*0.279*	*0.036*	*0.114*

to the relatively small sample of periodontally healthy subjects and the resulting low negative predicitive values, these data regarding the ability of the dot-blot assay or ELISA to delineate periodontal status did not reach a level of statistical significance.

IV. DISCUSSION

Peripheral capillary blood is used for a variety of laboratory tests. However, its use in determining elevated antibody to periodontopathic organisms has not been previously reported. Recently, two authors reported the use of blood collected by finger-stick to assess antibody titers to *B.gingivalis* in patients with periodontal disease (Mouton *et al.* 1987, Murray *et al.* 1989). After blood collection, the samples were centrifuged to separate the serum. All assays for antibody to *B.gingivalis* were then performed using this serum. The current research utilized whole capillary blood, thus simplifying the technique and reducing time and cost for sample preparation. The correlation between serum and capillary titers was found to be highly significant.

Of course, the presence of other blood elements would be expected to reduce the antibody reactivity in a given volume of capillary blood. Indeed, the reactivity in peripheral capillary blood was found to be consistently less than that in serum from the same patient. The mean capillary titer to *A.actinomycetemcomitans* was 47% of the mean serum titer to this organism. Capillary reactivity to *B.gingivalis* and *B.intermedius* averaged 68 and 48.8% of the serum titers respectively. Overall, the mean capillary reactivity was 54.6% that of serum.

The hematocrit is a measure of the number and size of cells present in the blood. The normal hematocrit for females is 40-45% and for males is 45-50%. Thus, a given blood sample consists of 40-50% cells. One would therefore expect that, for a given patient, the antibody reactivity in a sample of whole peripheral capillary blood would be approximately 50-60% that of an equal volume of serum. While there was variation in the percentage of capillary reactivity relative to serum reactivity among the subjects in this study, the mean capillary reactivity of 54.6% is in the range expected based on the use of whole blood samples. In order to compensate for the difference in reactivities, the dot-blot assay used positive and negative control sera at a 1:50 dilution to compare with the experimental capillary blood samples at a dilution of 1:25.

The use of whole capillary blood rather than serum introduces the possibility of error in sample collection. Some laboratories recommend against the use of finger-stick blood for red and white cell counts due to difficulty in standardizing capillary blood flow.

Furthermore, squeezing the finger to obtain blood will alter the composition of the blood specimen. Capillary blood obtained from an inadequate digi-puncture wound may yield lower cell counts than those obtained from venous blood due to dilution of the specimen with tissue fluid when the puncture site is squeezed (Bauer 1982). This may account for some of the variability in capillary antibody titers relative to serum titers in some of the subjects. Every effort was made during specimen collection to produce an adequate puncture wound which allowed a free flow of blood. Infrequently, a second puncture was necessary to obtain an adequate sample volume. In order to have a specimen volume adequate for several experiments, 60µl of capillary blood was collected from each subject. Since only 300µl of capillary blood at a 1:25 dilution was needed to adequately saturate the filter paper strips, a specimen of only 12µl in volume would be necessary for a single test. This should not require more than one finger- stick.

In developing the dot-blot assay, it was found that several steps were critical in securing accurate and readable results. Other steps allowed wider latitude without significant effect on the results. Antigen concentration was quite important. Excessive dilution of the organisms yielded numerous false negative results while inadequate dilution produced false positives. Conversely, dilution of the capillary blood samples was less critical, with minor alterations in dilution having little effect on distinction between positive and negative reactions in the dot-blot assay. When the paper strips containing capillary blood were placed over the antigen dots on the nitrocellulose, it was critical that all air be removed from between the strips and the nitrocellulose. Trapped air prohibited incubation of the antibody-containing fluid with the antigen dots, resulting in a lack of color change in part or all of the dot. The substrate reaction time was also very important. Reaction times of greater than three minutes resulted in heavy non-specific staining of the nitrocellulose and made results difficult, if not impossible to read. Incubation time of under two minutes produced numerous false negatives.

Positive control sera were selected which produced a strong color change in the dot-blot assay. Negative control sera were specifically chosen based upon their low, yet still visible reactivity. A negative control serum with no visible reactivity would cause even the weakest color change from an experimental sample to be deemed a positive reaction,

even though the antibody level was well within the normal range. While many of the reactions in the dot-blot assay were obviously positive or negative, there were many dots which fell between the positive and negative controls. When the ELISA titers for these dots were analyzed, they were generally found to be close to the "cut-off" points for assessing elevated versus normal reactivity for that organism. That is, weakly positive or negative results in the dot-blot assay were usually associated with weakly positive or negative ELISA results.

Each nitrocellulose sheet had five to ten experimental blood samples plus a positive and negative control. Due to the difficulty in reading the dots with color intensity between that of positive and negative controls, it was critical that each dot be compared only with the positive and negative control on the same nitrocellulose sheet. Thus, inherent variabilities in experimental technique for a given nitrocellulose sheet would affect all the dots to an equal degree.

Qualitative dot-blot results for the three organisms in 34 periodontally diseased subjects were compared to the quantitative ELISA results for the respective organism and subject. The sensitivity of the dot-blot assay relative to the ELISA was 0.95, with a false negative rate of only 5%. Thus, 95% of positive ELISA results were also deemed positive by dot-blot. The specificity of the dot-blot assay relative to the ELISA was 0.83, with a false negative rate of 17%. That is, 17% of negative ELISA results were deemed positive by the dot-blot. In general, when a dot had a degree of color change falling between the positive and negative controls, the dot was assessed as positive. This maximized the sensitivity of the test, yet produced several false positive results. Overall, the relationship between the dot-blot and ELISA results was found to be highly statistically significant ($p<0.000001$).

The dot-blot assay was designed as a diagnostic test of a screening nature. As with most tests of this type, the assay emphasizes identification of subjects with elevated antibody titers so that these individuals might then be examined further from a clinical and/or laboratory standpoint. Therefore, one is willing to accept a higher false positive rate while seeking to identify the greatest number of patients with truly elevated antibody titers. Thus, patients who have normal antibody titers may, on occasion, be identified by

the dot-blot as having elevated reactivity. Further work-up of the patient would reveal their true normality. However, it is unlikely that patients with truly elevated antibody titers would have negative dot-blot results.

In assessing the sensitivity and specificity of the dot-blot assay relative to its ability to identify elevated antibody titers to *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius*, the ELISA was used as the "gold standard". Unfortunately, the ELISA is not a perfect assay and does not always provide precise quantitative data. Ebersole et.al (1983) have demonstrated that antibody reactivity in replicate ELISA measurements may deviate up to 12% from the mean titer. This has significant applicability to the dot-blot results in the current study. If a given subject had a weakly positive dot-blot reaction to *A.actinomycetemcomitans* and an ELISA titer of 96 EU to that organism, the result would be a false positive finding ("cut-off" for *A.actinomycetemcomitans* = 100 EU). However, the ELISA titer may actually range from 84-108 EU (96 $\pm$ 12 EU). Thus, this individual may indeed have an elevated antibody titer to the organism, in which case the dot-blot result would be correct.

Because the dot-blot assay provides qualitative data, it is more difficult to makes concrete assessments of weakly positive or negative reactions. The ELISA, on the other hand, lends itself to more precise interpretation due to its quantitative nature. Thus, an ELISA titer one or two EU above the "cut-off" point is deemed positive, while a titer several EU below the "cut-off" is negative. It is much more difficult to decide whether a dot-blot result in this "gray area" is truly positive or negative since such decisions are based on visual assessments of qualitative color changes. In reality, both the ELISA and the dot-blot are subject to variability which may significantly affect the determination of elevated versus normal titers in this weakly positive/negative range of reactivity. Furuya *et al.* (1984) utilized a similar dot-blot assay and reported reactivity as both quantitative and qualitative data. Qualitative results were established visually as in the current study. Quantitative results were obtained by reading the colored dots spectrodensitometrically. With presently available technology, such spectrodensitometric measurement is cumbersome and generally not applicable to e in a clinical setting.

The dot-blot assay developed in the current study was shown to strongly reflect the

quantitative antibody titers determined by ELISA. In the past two decades, use of the ELISA has become widespread. It has been applied extensively in the laboratory setting to the study of systemic and local antibody responses to putative periodontopathogens. However, its use in the clinical setting is burdensome due to the cost of necessary equipment and reagents. Furthermore, the assay requires two days to run and is not cost efficient unless multiple patient samples can be analyzed at the same time. The ELISA is also somewhat technique sensitive and requires a trained individual to achieve accurate and reproducible results.

The dot-blot assay reported here has several advantages over the ELISA. Sample collection is simplified, requiring only a finger lancet and capillary tube. Laboratory centrifuges are quite expensive, and even the microcentrifuges used to remove serum from capillary blood samples cost several hundred dollars. With whole capillary blood, centrifugation is not necessary. The digi-puncture technique is also much easier than venous blood collection. Due to a lack of accessible veins, venipuncture is a laborious and exacting task for many patients. It is rarely difficult, however, to obtain a small capillary blood specimen from a finger-stick. For those patients with inaccessible veins, digi-puncture may significantly decrease the discomfort of blood collection. Likewise, not all clinicians are proficient in venipuncture; yet the finger-stick is easily mastered.

Use of the dot-blot assay greatly diminishes the equipment and reagent cost of performing the test, making it more conducive to clinical use. In addition, the time required to obtain results is dramatically reduced. The nitrocellulose sheets can be prepared well before they are needed. Following incubation with the antigens and blocking of non-specific reaction sites, the nitrocellulose can be dried and stored for later use. After the capillary blood specimen has been collected and diluted, the time required to perform the assay is just over two hours, rather than two days for the ELISA. Incubation of the antigens dots with capillary blood requires 60 minutes. This is followed by 15 minutes of washing. The peroxidase-conjugated anti-human IgG is then incubated for 30 minutes followed by another 15 minute wash. The nitrocellulose is then placed in substrate for 2 minutes, after which the results can be read.

The dot-blot assay can be performed for a single patient sample if desired, or the

capillary blood specimens can be stored in a freezer until multiple samples are run. Thus, if several subjects were tested on a single day, the assay could be accomplished at the end of the day. Conversely, if only a single patient were to be tested, results could be determined just two hours after sample collection.

The dot-blot assay may have a wide variety of applications in the clinical environment. Numerous studies have demonstrated elevation in antibody titers to one or more specific organisms in different categories of periodontal disease. The clinical and radiographic features of the periodontal diseases are often enough to make a diagnosis based on commonly used criteria. However, determination of elevated antibody to various organisms may provide a needed clue if diagnostic problems arise. The dot-blot assay provides a rapid and inexpensive means of obtaining another piece of the diagnostic puzzle.

The assay might prove useful in determining a periodontal prognosis for a given patient. Ranney *et al.* (1982) suggested a protective role for antibody to *A.actinomycetemcomitans* in patients with juvenile periodontitis. The presence of systemic antibody to the organism was inversely related to the severity of periodontal destruction. The number of periodontally involved teeth was significantly lower for those subjects with detectible antibody to *A.actinomycetemcomitans* than for those without antibody. A patient presenting with clinical and radiographic features consistent with the localized form of juvenile periodontitis might be tested for antibody to *A.actinomycetemcomitans* using the dot-blot assay. Absence of antibody to this organism may suggest a potential for generalization of the periodontal destruction.

Ebersole *et al.* (1982b) categorized the antibody response patterns in patients with various forms of periodontal disease. They then compared the risk of developing active disease with the antibody categories of the subjects (Ebersole *et al.* 1984a). Subjects with elevated antibody to three or more of the gram-negative species tested (category VI) responded poorly to periodontal therapy and continued to have significantly higher rates of disease activity than all other subjects. This suggests that subjects with elevated antibody to multiple putative periodontopathogens may have a less favorable prognosis both before and after treatment. The dot-blot assay may be useful in identifying patients

with such antibody responses and may suggest modification of treatment regimens to provide more aggressive therapy. In addition, the patient could be informed of the less than favorable prognosis at the outset of treatment.

Dot-blot and ELISA data from 34 periodontally diseased and 10 periodontally normal subjects were examined for their ability to predict the periodontal status of the subjects, i.e., "diseased" or "healthy". Using the panel of three organisms, the overall sensitivity of both the ELISA and the dot-blot was 0.68. Thus, 68% of periodontally diseased subjects had an elevated response to one or more of the three organisms. The overall specificity was 0.60; 60% of periodontally healthy subjects had a negative response to all three organisms. The relatively low specificity was related primarily to the presence of elevated antibody to *B.gingivalis* in 3/10 periodontally healthy subjects. One of these individuals was a third year dental student while another was a dental hygienist. It is possible that occupational inoculation of these individuals resulted in production of antibody to *B.gingivalis* in the absence of periodontal destruction. Ebersole *et al.* (1982b) determined that up to 15% of periodontally healthy individuals possess elevated antibody to one or more organisms associated with periodontal disease. Only 1/10 healthy subjects in the current study had elevated antibody to *A.actinomycetemcomitans* or *B.intermedius* by dot-blot. This compares favorably with the 15% incidence of elevated antibody in healthy patients reported by Ebersole. The 10% incidence of antibody to *A.actinomycetemcomitans* in the current study also correlates well with the 10% incidence of infection with this organism in periodontally normal subjects (Zambon *et al.* 1983a).

No attempt was made in this study to delineate the type of periodontal disease diagnosed in the 34 diseased subjects. It is likely that the sensitivity and specificity of the ELISA and dot-blot relative to disease status would be improved by focusing on specific periodontal diagnoses. For instance, 14/34 periodontally diseased subjects had an elevated dot-blot response to *A.actinomycetemcomitans* (sensitivity = 0.41). Elevated antibody to *A.actinomycetemcomitans* is common in LJP, but much less common in other forms of disease. Genco *et al.* (1985) used ELISA to demonstrate that 71% of LJP subjects had elevated antibody reactivity to this organism. The greater frequency in the report by

Genco *et al.* is directly related to the fact that a subject population was chosen which had a high probability of possessing elevated antibody to a single target organism. In the current study, the subjects represented a broad range of periodontal diagnoses.

Dot-blot responses to each of the three individual organisms were analyzed for their ability to identify the periodontal status of the subjects. The low sensitivity (0.11-0.47) of each individual organism is expected because periodontally diseased subjects may not have elevated antibody to that particular organism. If *A.actinomycetemcomitans* was the only organism tested, only 41% of diseased subjects would have a positive dot-blot response. However, when the panel was expanded to include three organisms, the sensitivity increased to 0.68. Thus, 68% of diseased patients had a positive response to one or more of the organisms on the panel.

It is highly probable that inclusion of additional periodontopathic organisms in the panel would improve the predictive capacity of the dot-blot test. Using the ELISA, Ebersole *et al.* (1985) found that 96% of subjects with various forms of periodontal disease had elevated ELISA antibody titers to one or more of a panel of 18 organisms. The panel of organisms for the dot-blot assay was recently expanded to include six organisms, the three previously used plus *E.corrodens*, *W.recta*, and *F.nucleatum*. Of 57 periodontally diseased subjects, 52 had a positive response to one or more of the organisms (Ebersole, personal communication of unpublished data). Thus, increasing the number of organisms on the panel improved the detection of diseased individuals.

When evaluating the predictive capabilities of any diagnostic test, two of the most important statistics are the positive and negative predictive values of the test. When the dot-blot assay is used to delineate diseased from healthy individuals, the positive predictive value answers the question, "How many of the patients with a positive dot-blot result will actually be periodontally diseased?" The negative predictive value describes the number of subjects with a negative dot-blot result who are truly periodontally healthy. Using *A.actinomycetemcomitans*, *B.gingivalis*, and *B.intermedius* as test organisms, 85% of subjects in this study population with a positive dot-blot response to one or more than one of the organisms were found to be periodontally diseased. Consequently, the dot-blot assay was able to predict with an accuracy of 85% that an individual subject was

periodontally diseased using three bacteria. Using these same organisms, only 35% of subjects with a negative dot-blot response to all three organisms on the panel were periodontally healthy. This low negative predictive value is expected since many periodontally diseased individuals have normal antibody titers to these three organisms. More importantly, this study population was heavily skewed toward disease.

The positive and negative predictive values of any diagnostic test are highly dependent upon the prevalence of the disease in question (Griner *et al.* 1981). In general, as the prevalence of disease in the population increases, the predictive value of a positive test increases, while the predictive value of a negative test decreases. In the current study, the prevalence of disease was 77.3% (34/44); likewise, the positive predictive value was quite high at 85%. The negative predictive value was low at only 35%. Obviously, the prevalence of disease in this study sample does not necessarily reflect the prevalence of periodontal disease in larger populations. Therefore, no conclusions can be drawn as to the value of systemic antibody determinations via the dot-blot or ELISA relative to detection of a periodontally diseased state in populations other than that currently under study.

The purpose of analyzing the ability of the dot-blot assay to delineate periodontal status was not to suggest that this assay be used to identify patients with overt periodontal disease. Identification of a periodontally diseased state is best done by combinations of clinical and radiographic instrumentation. There is no need or desire to replace these diagnostic tools, as they are readily available and relatively easy to use. While the dot-blot assay may not be needed to identify periodontitis in those patient populations who already manifest the disease, all patients with periodontal disease were periodontally healthy at some stage of life. The dot-blot assay may be useful in identifying individuals who are clinically normal at present, but may be at risk of developing periodontal disease in the future. Zambon *et al.* (1983a) proposed that transmission of *A.actinomycetemcomitans* may be responsible, in part, for the familial pattern of LJP. They suggested that determination of systemic antibody to this organism may help identify family members at risk for developing the disease. Genco *et al.* (1985) found elevated antibody to *A.actinomycetemcomitans* in non-diseased siblings of LJP patients and

proposed that elevated antibody responses to the organism may precede clinical signs of LJP in these individuals. For those subjects who are potentially at risk for disease development, use of the dot-blot assay in the clinical arena may suggest more frequent and detailed examination is required, and may lead to early intervention.

The initial change from a state of periodontal health to one of disease may also be indistinct. Early disease may present as 4-5mm probing depths with minimal radiographic bone loss. There is significant inter- and intra-examiner variation both in probing force (Freed *et al.* 1983) and in probing depth measurements (Aeppli *et al.* 1985), and the initial change from health to disease may not be accurately assessed by multiple examiners. When patients present with clinical signs of suspected early periodontal involvement, the dot-blot assay may be useful in assessing the patients' risk for disease and is an adjunct in confirming the tentative diagnosis.

Future research should be directed toward longitudinal monitoring of clinically normal subjects who demonstrate a positive dot-blot to one or more organisms (i.e., false positives). It is possible that some of these "normal" patients will develop clinical signs of disease several months or years after initial examination. This may indicate the presence of a subclinical periodontal infection in these subjects at initial presentation. It would be interesting to analyze the eventual fate of subjects who are deemed "at risk" by the dot-blot assay but who show no evidence of disease at the time of evaluation.

It could be argued that the presence of elevated antibody to a given organism does not necessarily indicate an existing infection with the same organism. Ebersole *et al.* (1984a, 1987b) have demonstrated an 85% agreement between elevation of systemic antibody to a given organism and the intraoral colonization by the same bacteria. Moreover, in those subjects with elevated antibody titer to a given organism, that organism was found in 55% of disease-active sites compared to only 18% of disease-inactive sites. This suggests that elevation in systemic antibody to organisms associated with the periodontal diseases generally indicates intraoral colonization by those organisms. Similar research in a more controlled study revealed that 80% of subgingival plaque samples from disease active sites possessed the same organism to which the subject showed an elevated antibody titer, while only 20% of disease-inactive sites

possessed the organism (Ebersole *et al.* 1987a).

Genco *et al.* (1985) examined the specificity of the systemic antibody response in LJP patients. Subjects infected with *A.actinomycetemcomitans* serotype a demonstrated a specific elevation in antibody to only this serotype, while those infected with serotype *b* had antibody only to serotype *b*. This supports the concept of a specific antibody response which is reflective of oral colonization by a given organism.

The presence of an elevated antibody response does not always correlate with the concomitant presence of the homologous organism (Morinushi *et al.* 1989). There may be several explanations for this disparity. Most studies utilize subgingival plaque samples from selected sites in the oral cavity. It is possible that, while the sampled sites may not harbor the organism to which an elevated antibody response is seen, other unsampled sites may. Particularly virulent organisms may stimulate a humoral immune response at low levels, perhaps prohibiting successful detection of the organism with currently available techniques. Potentially pathogenic organisms may become prominent in the subgingival plaque only in acute episodes, impeding their detection at stages between these short intervals. Finally, reduction or elimination of an organism as a consequence of therapy does not result in an immediate reduction in systemic antibody titer. This may be due to the time required for elimination of circulating antibodies and/or the maintenance of an anamnestic response by undetectable levels of the organism/antigen remaining in diseased or non-diseased sites. While the presence of antibody to a given organism does not necessarily indicate presence of that organism in diseased sites, it is a definite indicator of past or present colonization by the organism at some site. A positive response to one or more organisms in the dot-blot assay may encourage the clinician to provide more aggressive therapy directed at detection, reduction, or elimination of those organisms.

Aukhil *et al.* (1988) have suggested the analysis of antibody titers while monitoring patients in the maintenance phase of treatment. Due to the sensitive anamnestic response, subjects who have mounted an antibody response to putative periodontopathic organisms may exhibit a rapid rise in antibody titer should colonization with the same organism occur following therapy. The dot-blot assay may be useful in monitoring the

systemic antibody patterns during maintenance. Positive dot-blot results during this period may suggest the need for further examination and treatment.

The dot-blot assay may thus be a valuable tool for assessment and monitoring of patients throughout therapy. It may be used to help identify patients at risk for developing periodontal disease. It may aid in determining the diagnosis and prognosis of the disease process. Likewise, the assay may suggest treatment alterations and may help monitor the results of therapy. In addition to its use in direct patient care, the dot-blot assay may prove valuable for clinical or epidemiologic research. It is a relatively inexpensive test and is simple to perform, thus reducing the time and cost involved in laboratory determination of antibody reactivity. The assay can be run with a single blood sample or a large number of specimens, with minimal increase in the time required. Simplification of the procedures virtually eliminates the need for highly trained laboratory personnel. While several steps in the assay require attention to detail, it is well within the ability of any clinician to perform the test.

V. SUMMARY AND CONCLUSIONS

The determination of systemic antibody responses to putative periodontal pathogens is a common focus of research. Elevation in systemic antibody titer appears to represent a specific immune response to colonization by these organisms. Assessments of antibody responses have been performed as a means of identifying patients at risk for developing disease, clarifying periodontal diagnoses, categorizing responses to periodontal therapy, and altering the course of periodontal treatment.

Presently, determination of systemic antibody responses requires costly and time-consuming laboratory analysis. This has limited the usefulness of antibody assessment to the realm of research activities. The individual patient has received little benefit from this research, except in a tangential fashion. The clinician may know that certain antibody profiles are associated with specific diseases or that antibody analysis may aid in treatment of the patient. However, the availability and rapidity of antibody analysis is limited. Thus, most patients are never assessed regarding their antibody responses to periodontitis-associated organisms. The dot-blot assay greatly simplifies this assessment. It was determined that accurate antibody determinations can be performed using whole peripheral capillary blood. The qualitative results of the dot-blot assay correlate strongly with the quantitative data obtained by ELISA, the "gold standard" of antibody analysis. While the ELISA is a more precise method of determining antibody reactivity, the dot-blot provides a method of evaluating patients in a short period of time at relatively low cost. If dot-blot results suggest further study is indicated, patients can be referred for quantitative laboratory analysis via ELISA.

This research was primarily directed at groundwork development of a rapid, qualitative assay for determining antibody responses. As such, the technique is only in its preliminary stages of evolution. By addition of more organisms to the panel of antigens, the test will become more meaningful and applicable to a wider range of uses. Although two hours is a short time for achieving test results, alteration in reagents or reaction time may further shorten the interval from specimen collection to test completion. It is hoped that this research will be further refined and simplified in order to expand its use.

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VITA

Brian Luke Mealey was born in Houston, Texas on September 20, 1959 to George Alexander Mealey and Jeanne Cavanagh Mealey. Following graduation from Strake Jesuit College Preparatory in Houston, Texas in June 1977, he attended Texas A & M University in College Station, Texas. In August 1979, he was admitted to the Dental School at the University of Texas Health Science Center at San Antonio. In June 1983, he received the degree of Doctor of Dental Surgery and in July 1983, he began a General Practice Residency at the USAF Hospital, Barksdale AFB, Bossier City, Louisiana. In July 1984, he was assigned as a staff general dentist to USAF Clinic, Soesterberg AB, The Netherlands. In July 1987, he entered the Post-Doctoral Periodontics program at the University of Texas Health Science Center at San Antonio in conjunction with Wilford Hall USAF Medical Center.

Dr. Mealey was married to Carla Rhea Reddy in 1983. They have two children, Colleen Marie, born in 1986, and Patrick Luke, born in 1988.