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Appraisal of Biofilm Formation in Diabetic Foot Infections by Comparing Phenotypic Methods With the Ultrastructural Analysis

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ABSTRACT

Diabetic patients are more prone to the development of foot ulcers, because their underlying tissues are exposed to colonization by various pathogenic organisms. Hence, biofilm formation plays a vital role in disease progression by antibiotic resistance to the pathogen found in foot infections. The present study has demonstrated the correlation of biofilm assay with the clinical characteristics of diabetic foot infection. The clinical characteristics such as the ulcer duration, size, nature, and grade were associated with biofilm production. Our results suggest that as the size of the ulcer with poor glycemic control increased, the organism was more likely to be positive for biofilm formation. A high-degree of antibiotic resistance was exhibited by the biofilm-producing gram-positive isolates for erythromycin and gram-negative isolates for cefpodoxime. Comparisons of biofilm production using 3 different conventional methods were performed. The strong producers with the tube adherence method were able to produce biofilm using the cover slip assay method, and the weak producers in tube adherence method had difficulty in producing biofilm using the other 2 methods, indicating that the tube adherence method is the best method for assessing biofilm formation. The strong production of biofilm with the conventional method was further confirmed by scanning electron microscopy analysis, because bacteria attached as a distinct layer of biofilm. Thus, the high degree of antibiotic resistance was exhibited by biofilm producers compared with nonbiofilm producers. The tube adherence and cover slip assay were found to be the better method for biofilm evaluation.

Diabetic foot infections are among the most feared complications and the leading cause of hospitalization of patients with diabetes (1). The incidence of patients undergoing foot amputations has been increasingly in recent years (2). Foot ulcers are a complex polymicrobial community, in which *Staphylococcus aureus* is a dominant early colonizer of wounds, together with *Enterococcus* spp., *Corynebacterium* spp., and coagulase-negative Staphylococci. These species are followed by *Pseudomonas* and various members of the Enterobacteriaceae (3). Consequently the term "biofilm" refers to these microbial populations that are attached to a surface or to the surfaces of other organisms and encase themselves in a hydrated extracellular polymeric substance (EPS), also referred to as "slime." The chemical and physical properties of the EPS vary, but it is mainly composed of thick polysaccharides layers (4,5). This EPS attaches within minutes and forms strongly attached microcolonies within 2 to 4 hours. These develop the initial EPS and within 6 to 12 hours evolve into fully mature biofilm colonies that are extremely resistant to biocides. These fully mature biofilm colonies shed planktonic bacteria within 2 to 4 days. Also, depending on the species and growth conditions, the EPS can rapidly recover from mechanical disruption and reform mature biofilm within 24 hours (6). The EPS is also associated with other macromolecules such as proteins, DNA, lipids, and, even, humic substances (7).

This biofilm formation is predicted to be one of the most important virulence factors in foot infections (8). It protects the organisms from phagocytosis and facilitates antibiotic resistance, acting as a barrier and decreasing the diffusion of antibiotics, antimicrobial proteins, lysozyme, and small molecular antimicrobial agents such as defensins (9). Examination of the culture and molecular results is needed to compare and contrast the abilities of different methods to detect bacteria. A potential limitation of molecular diagnostics is the inability to evaluate phenotypic antibiotic sensitivity (10). Oliveira and de Lourdes RS Cunha (11) reported that the sensitivity and specificity of the phenotypic methods were 100%. However, the phenotypic method could be imprecise compared with molecular analysis of the genes involved in biofilm production.

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The development of biofilm in the wound makes it severe and complicates the treatment process, thereby delaying the healing process and resulting in the development of drug-resistant strains (12,13). Most antimicrobial therapies for these biofilms have largely proven unsuccessful, because the mechanism of biofilm-associated antibiotic resistance is multifactorial (14). The most effective treatment of biofilm infections has been physical removal, followed by inhibition of biofilm reconstitution with antibiofilm agents and antibiotics (15). However, patients who develop chronic wounds are often immunocompromised, which facilitates the establishment of bacterial biofilm communities and makes elimination of the biofilm even more difficult (16). Therefore, a better understanding of bacterial biofilms is very much needed in the treatment of these infections. In southern India, only 1 study has evaluated the biofilm as a major virulence factor in diabetic foot infections (17). Although 1 or 2 studies have evaluated biofilm formation using conventional methods, no studies have evaluated the biofilm as a major virulence factor in large population of the patients to arrive at a definite conclusion on the nature and type of biofilm produced. Hence, the objective of the present study was to assess the biofilm as a major virulence factor in patients with diabetic foot infections using 3 different conventional phenotypic methods compared with ultrastructural analysis using scanning electron microscopy (SEM).

Patients and Methods

During a 1-year period, 160 patients with diabetic foot infections presenting to the outpatient department of the diabetic care center in Salem, Tamilnadu, India (Fig. 1) were enrolled in the present study. The institutional ethical committee approved the present study for the enrollment of human subjects under clinical evidence level II. The study participants provided written informed consent before undergoing sampling. The clinical history of these patients, such as the duration of diabetes, type of diabetes, ulcer site, smoking habits, duration of the hospital stay, and other associated comorbid conditions, were recorded. Clinical examination of the ulcer was performed using the Wagner grade classification (18). Swabs were taken from the ulcer using a rotatory movement with the swab and were processed for routine microbiologic analysis. The samples were processed by inoculation on culture media, such as sheep blood agar, brain heart infusion agar, or nutrient agar, and incubated at 37°C for 24 hours. The bacterial isolates grown on the media were confirmed using *Bergey's Manual of Determinative Bacteriology* (19). Antibacterial susceptibility testing was performed using the method provided in the Clinical Laboratory Standards Institute 2012 guidelines (20). The antibacterial discs included in the present study were erythromycin (15 μg), amikacin (30 μg), chloramphenicol (30 μg), clindamycin (10 μg), oxacillin (1 μg), cefoxitin (30 μg), tetracycline (30 μg), and ciprofloxacin (5 μg) for gram-positive organisms. Aztreonam (30 μg), amoxyclav (30 μg), cefpodoxime (10 μg), cefepime (30 μg), cefoperazone (75 μg), cefoperazone/sulbactam (75/10 μg), cefixime (5 μg), piperacillin (100 μg), ceftriaxone (30 μg), amikacin (30 μg), rifampicin (5 μg), meropenem (10 μg), cefoxitin (30 μg), ceftazidime/clavulanic acid (30/10 μg), ticarcillin/clavulanic acid (75/10 μg), and piperacillin/tazobactam (100/10 μg) were used for the gram-negative organisms.

Tube Adherence Method

The biofilm formations of the diabetic foot isolate were examined using the tube adherence method described by Christensen et al (21).

Congo Red Agar Method

The determination of biofilm formation using the Congo red agar plate method was performed following the method described by Freeman et al (22).

Liquid Interface Cover Slip Assay

The biofilm formation of the diabetic foot isolates was also determined using the air–liquid interface cover slip assay, following the method described by Mathur et al (23). Strains of *Staphylococcus aureus* MTCC 96 and *Pseudomonas aeruginosa* MTCC 424

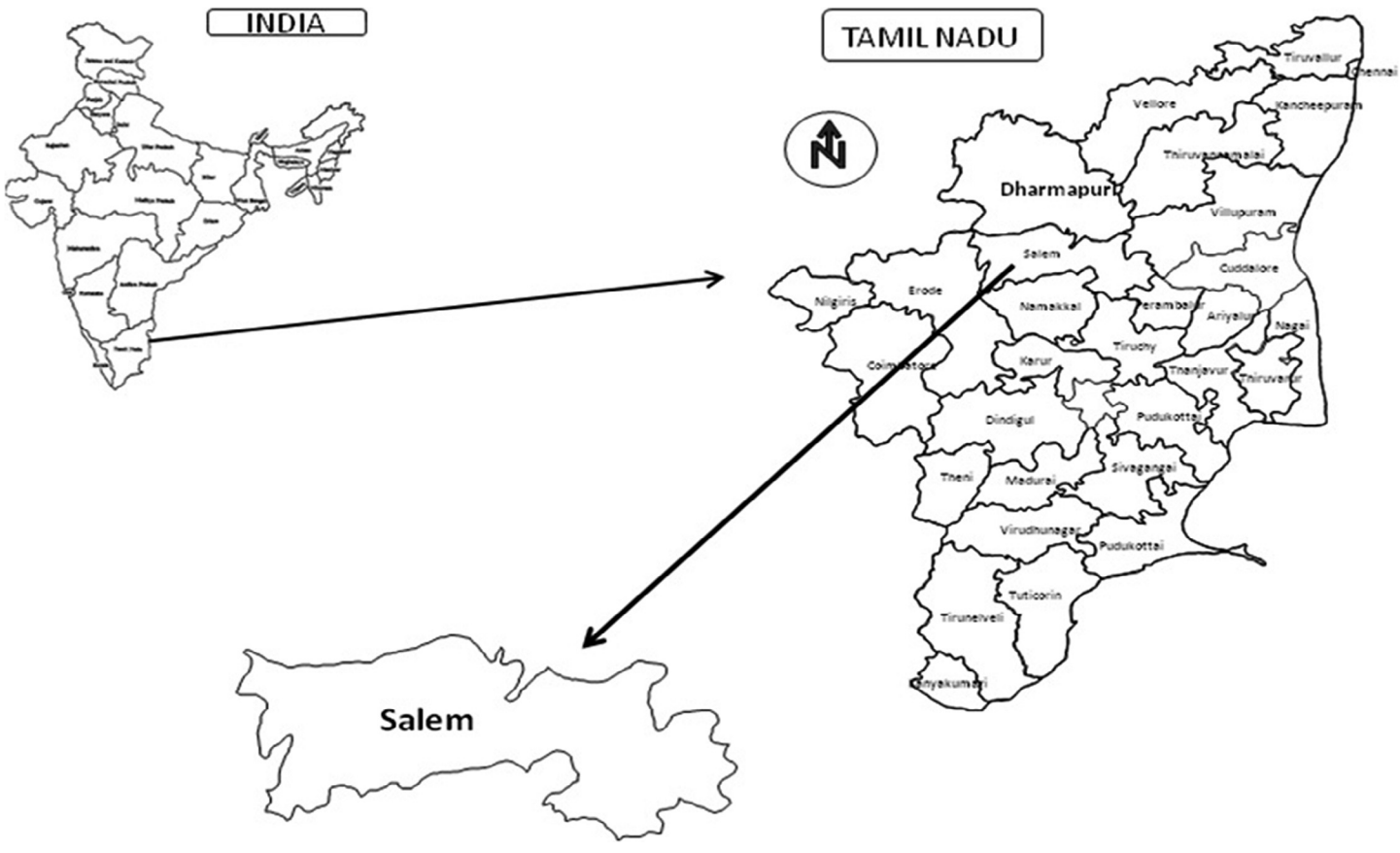

Fig. 1. Location of sample collection area.