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Original Article

Molecular and culture based assessment of bacterial pathogens in subjects with diabetic foot ulcer

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ABSTRACT

Introduction: Expeditious and precise discerning of bacterial pathogens is a fundamental grail, of clinical diagnostic microbiology. Genotypic detection is a budding substitute to recognize phenotypic culture based processes in bacterial identification.
Aims: We report a comparative evaluation of biochemical and genomic-based assays for exploring the commonest bacterial flora of infected diabetic foot ulcers along with clinical variables of subjects enrolled.
Methods: The pathogens selected (i) *Staphylococcus aureus* ii) *Pseudomonas aeruginosa,* iii) *Escherichia coli* and iv) Klebsiella pneumonia, stood for the most frequent isolates of diabetic foot infection in previous studies from Northern India. Identification of these pathogens were done by conventional assays and polymerase chain reaction.
Results: Of 50 specimens obtained from infected DFUs, 74% of cases were affirmative by bacteriological assays and 90% showed positivity via polymerase chain reaction (PCR). Among processed samples 44 isolates were detectable through phenotypic analysis and 65 bacteria by species-specific PCR. Thirteen samples and 21 isolates could not be scrutinized by phenotypic identification systems. The most prevalent pathogens identifiable were *Klebsiella pneumonia,* followed by *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli.*
Conclusions: We have shown that PCR-based diagnostic methods improved the identification compared to conventional methods and highlight the incorporation of PCR due to shorten turnaround time translating into improved clinical outcomes.

1. Introduction

Diabetes mellitus is imposing a major burden on developing countries and hence considered as a global epidemic of the 21st century. According to world health organization (WHO), the top 10 countries with the greatest number of diabetics are India, China, USA, Indonesia, Japan, Pakistan, Russia, Brazil, Italy, and Bangladesh. Diabetic foot ulcers followed by infection is a serious secondary complication of diabetes. The lifetime risk for a diabetic to develop DFU is about 15–25% [1]. Diabetic foot ulcers (DFUs) are a cause of complicated combination of various intrinsic factors, peripheral neuropathy, peripheral arterial diseases, deformity in foot, poor extremity perfusion, and extrinsic causes such as high plantar pressures [2]. DFU in developing countries is a major cause of morbidity, hospital admissions and associated mortality.

In diabetes poor wound healing due to compromised blood supply to superficial and deep structures, sugar coated micro-vasculatures along with impaired host immune responses and unnoticed lesions provides a niche for infection [3]. Infected DFUs span the spectrum from simple, superficial cellulitis to chronic osteomyelitis ultimately leading to dreaded complications like gangrene, systemic toxicity and lower limb loss [4]. The diversity of bacterial populations in diabetic wounds is an important contributor to the chronicity of wounds. Microbes harboring the wound are often present in multi-layers forming biofilms, encasing themselves in self-produced hydrated extracellular polymeric substance (EPS), which is also referred to as “slime” which provides a shield from antimicrobial agents impairing wound healing [5].

Exploring the spectrum of microbes invading diabetic lesions is central which involves the proper swab specimens obtained from wound after debridement. Infection is routinely confirmed by standard conventional bacteriological tools [6]. From past many

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decades, a culture of a wound specimen was the only way to determine the causative pathogen (s) in a DFI. Often fastidious growing organisms are not identified providing biased and delayed results by using traditional bacteriology protocols. In the past few years, however, molecular microbiology techniques (direct PCR, 16 ribosomal DNA sequencing, denaturing gradient gel electrophoresis, pyro-sequencing, etc) have demonstrated the presence of greater numbers as well as varieties of species in various types of wounds than had previously been documented [7]. Tools of molecular genetics are advancing, and microbial diversity of wounds can be more easily and sensitively explored by using molecular techniques than culture methodologies [8].

One of the modern techniques for identifying pathogens relies on PCR amplification assays with specifically designed nucleotide primers. PCR is suggested to be safer, accurate and more rapid technique then ordinary bacteriology methods for diagnosing viruses and bacteria [9]. Molecular microbiological assays have uniformly verified that most DFU serves host for many more bacterial species than were previously detected based on the results of standard microbiological cultures [10].

We hypothesize that use of polymerase chain reaction for microbial identification directly on DNA extracted from pus specimens of wounds will provide a broad spectrum of isolates than culture-based methodologies. In the present study, both conventional and molecular diagnostic (PCR) techniques were used for analyzing pus specimens collected from infected diabetic foot wounds, for the presence of the four common bacterial pathogens of DFUs specified in previous studies of same region [2]. The aim of this study is to characterize the aerobic pathogens of diabetic foot wounds using conventional based and molecular techniques.

2. Material methods

2.1. Clinical examination

A prospective hospital-based study was conducted in Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India during the period from July 2014 to March 2016. Fifty type 2 diabetic patients with infected foot ulcers admitted to endocrinology ward were enrolled in study. All the subjects gave informed consent, and clearance was obtained from the Bio-Ethical Committee (BEC), F/O Medicine, J.N. Medical College, Aligarh Muslim Univversity, Aligarh, India.

A detailed history and physical examination was done on every subject. Age, sex, body mass index (BMI), duration of diabetes, glycated hemoglobin (HbA1c), duration of diabetes, presence of retinopathy (funduscopy), hypertension, nephropathy (creatinine >1.5 mg% or presence of micro or macro-albuminuria), peripheral vascular disease (ischaemic symptoms and intermittent claudication of rest pain, with or without absence of pedal pulses or posterior tibial pulses), neuropathy (absence of perception of the Semmes–Weinstein monofilament at 2 of 10 standard planter sites on either foot) and clinical outcomes were noted in every patient. Clinical assessment of infection in wound was made on presence of classic signs of inflammation (redness, swelling, tenderness, warmth, or pain) or purulent secretions and additional or minor signs of non-purulent secretions, friable or discolored granulation tissue, undermining of wound edges, foul odor. Size of ulcer was calculated by multiplying longest and widest diameters and mentioned in centimeter square. Ulcers were graded using Wagner's classification system grade I (superficial ulcer or subcutaneous tissue), grade II (ulcers extended into tendon, bone, or capsule), grade III (deep ulcer with osteomyelitis, or abscess), and grade IV (gangrene of toes). Subjects with grade 0 (uninfected lesions/intact skin/healed ulcers) were excluded from the study. Informed consent was given by each patient, and the study was approved by Institutional ethics committee (IEC).

2.2. Conventional analysis

Pus aspirates or soft tissue samples were collected on the day of admission after proper cleaning of wound with saline followed by debridement and immediately processed for aerobic bacterial identification to avoid less accurate results. The specific detection of bacterial pathogens was based on microscopic morphology, staining characteristics, culture and biochemical properties using standard laboratory criteria.

2.3. Molecular analysis

2.3.1. DNA extraction

The pus sample was obtained from debrided wound using a sterile swab stick. The specimen was immediately inoculated in sterile nutrient broth and allowed to grow at 37 °C for 18 h. The pelleted broth was processed for isolation of DNA using commercially available DNA extraction kit (Sigma-Aldrich, India) according to manufactures instructions. DNA samples were stored in elution buffer at −20 °C until analysis.

2.3.2. PCR primers

Oligonucleotide primers for the PCR are shown in Table 1

2.3.3. Detection of aerobic bacteria by PCR

Five micro liters (200 ng) of the prepared DNA template was added to 45 ml of PCR reaction mixture in 0.2 ml thin walled microcentrifuge tube. The reaction mixtures used in the PCR steps contained 1 X PCR buffer, 2.0 mM $MgCl_2$, 0.1 mM of each deoxynucleoside triphosphate, 1.0 mM of forward primer, 1.0 mM of reverse primer (as indicated in Table 1), and 2 U of Taq DNA polymerase.

2.3.3.1. Cycling conditions. PCR was carried out in thermal cycler with first cycle of denaturation at 95 °C for 15 min, 35 cycles of

Table 1
Sequences of primers used in PCR.

Microorganisms	Primer sequences	Tm (°C)	Product size (bps)	Ref No.
Staphylococcus aureus	F-GATTGATGGTGATACGGT R- CAAGCCTTGACGAACTA	57	273	[7]
Klebsiella pneumoniae	F-CAACGGTGTGGTTACTGACG R-TCTACGAAGTGGCCGTTTTC	55	108	[11]
Pseudomonas aeruginosa	F-GGGGGATCTTCGGACCTCA R-TCCTTAGAGTGCCCACCCG	58	956	[12]
Escherichia coli	F-CCGATACGCTGCCAATCAGT R-ACGCAGACCGTAGGCCAGAT	55	884	[13]

F, forward primer; **R,** reverse primer; **Tm**, annealing temperature; **bps,** base pairs.

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