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Association of bacteria in diabetic and non-diabetic foot infection — An investigation in patients from Bangladesh

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Summary The microbial community on a host relies on its immune status and pathophysiological condition. *Diabetes mellitus* is a metabolic disorder associated with a 25% increased risk of developing foot infection. The pathophysiological differences between diabetic foot infection (DFI) and non-DFI patients may alter the microbial composition in infections. The present study aims to comparatively analyze the microbes colonized in DFI and non-DFI patients in Bangladesh. Pus specimens were collected from 67 DFI and 12 non-DFI patients to investigate the bacteria associated with foot infection. For this investigation, an array of microbiological, molecular biological and immunological approaches were performed. Common bacteria detected in both DFI/non-DFI samples were *Pseudomonas* spp. (22/29%), *Bacillus* spp. (12/3%), *Enterobacter* spp. (22/7%), *Staphylococcus* spp. (13/13%) and *Acinetobacter* spp. (10/10%). *Enterococcus* spp. (9%) and *Klebsiella* spp. (8%) occurred only in DFI patients, whereas *Citrobacter* spp. (29%) was only detected in non-DFI samples. The rate of occurrence of three organisms, namely, *Enterococcus* spp. $|Z| = 2.2125$, *Klebsiella* spp. $|Z| = 1.732$, *Bacillus* spp. $|Z| = 1.9034$, were also statistically significant. Most of the isolates from DFI patients were commonly resistant to the cephalosporin (Ceftazidime, Ceftriazone, Cefurozime) and monobactam (Aztreonam) groups of antibiotics. DFI patients had comparatively higher C-reactive protein (CRP) levels than non-DFI patients, and a positive correlation was observed between multi-antibiotic resistance and CRP levels (one of the markers of chronic

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subclinical inflammation). The present investigation implicated a complex association of the bacterial population in DFI compared with non-DFI with different antimicrobial resistance properties, which was linked with CRP levels.
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Introduction

Diabetes mellitus (*DM*) is now a major health problem all over the world and is increasing globally at an alarming rate [1]. It has been declared an epidemic in developing countries, including Bangladesh [2]. Approximately 347 million people are suffering from *DM* worldwide, which is predicted to double by the year 2025 [3]. The prevalence of diabetes in Bangladesh is increasing rapidly, leading to complications of chronic diabetes. Diabetic foot infection (DFI) is one of the most serious complications of *DM*. Diabetic patients have a 25% increased risk of developing a foot ulcer [4]. The primary causes of DFI are microbial agents, and their early diagnosis is essential for appropriate antimicrobial therapy [5]. Once an infection has developed in DFI patients, it is difficult to treat because of impaired microvascular circulation to the lower limb, which limits the access of phagocytic cells and antibiotics to infected sites [6]. Common organisms reported in foot infection are mainly *Staphylococcus* spp. and *Enterococcus* spp. arising from the patient's own body [7]. Extensive tissue destruction and poor blood circulation are a result of infection with *Pseudomonas* spp., *Proteus* spp., and *Enterococcus* spp. bacterial groups [7]. The major predisposing factor associated with these infections is foot ulceration, which is usually related to peripheral neuropathy and peripheral vascular disease, and various immunological disturbances play a secondary role in the development of diabetic foot ulceration [8]. Chronic subclinical inflammation (CSI) reportedly has a significant association with the development of acute diabetic foot syndrome [9]. C-reactive protein (CRP) is an acute phase protein whose concentration increases in the blood in response to inflammation. CRP is a marker of CSI [10].

There is scant knowledge on the microbial composition inhabiting the DFI area and the correlation of these microbes to CSI. The present study was performed to determine the bacterial composition in foot lesions of DFI and non-DFI patients and their antibiotic resistance patterns. The investigation also explores the correlation of serum CRP levels with microbial composition and resistance properties. This study is expected to generate valuable information, which will be helpful in the management and prevention of foot infection in our population and will help clinicians to select and develop appropriate drugs.

Materials and methods

Specimen collection

Pus specimens were collected from infected foot wound sites of 67 DFI and 12 non-DFI patients from the Diabetic Foot Care Hospital (DFCH) and Dhaka Medical College Hospital (DMCH), respectively. All of the diabetic foot ulcers were included in this study irrespective of ulcer grading. According to the questionnaire prepared for this study, subjects suffering from diseases such as cancer, autoimmune diseases, cardiovascular diseases and renal diseases were excluded. Pus specimens were collected from the patients after ulcer base debridement. To avoid contamination, foot wounds and tissue debris were thoroughly cleaned with sterile normal saline followed by gentle rubbing of the wound site with 70% alcohol prior to swabbing the pus sample. Sterile cotton swab sticks were moistened with sterile normal saline before specimen collection. Then, the swab sticks were extended deeply into the depth of the lesion to avoid contamination from the wound surroundings. When copious volumes of pus existed, samples were collected aseptically by needle aspiration to avoid major exogenous contamination. After pus sample collection, the swabs were transported to the laboratory by immersion to maintain aseptic conditions (20-ml test tube containing 10 ml of peptone water). The samples were properly labeled and immediately transported to the laboratory for further investigation. Blood samples were also collected from these patients for investigation of subclinical inflammation. A detailed history was collected from each of the subjects and control patients, and the demographic data included age, sex, occupation, socioeconomic status, type of water used, and type of treatment used.

Determination of total viable *Enterobacteriaceae* and resistant bacteria counts

Sample tubes containing pus swabs (67 DFI and 12 non-DFI) in peptone water were incubated in a rotary shaker at 120 rpm at 37°C for 1 h. The collected pus samples were serially diluted up to 10^{-4} with sterile normal saline (0.85%). To determine the total viable count (TVC), the *Enterobacteriaceae* count (EC) and the resistant bacteria count (RBC), 100 μl of serially diluted pus samples were spread on Nutrient Agar (NA) plates, MacConkey Agar (MAC) plates and NA supplemented with Ciprofloxacin (CIP, 0.16 μg/ml), Cefotaxime (CTX, 1.0 μg/ml) or both. All of the plates were incubated at 37 °C overnight, and suspected colonies were purified on the same media plate and preserved in 20% glycerol-supplemented nutrient (NB) broth for further investigation.

Morphological and biochemical isolate identification

Microscope observations and gram staining were performed on the isolates. The morphological characteristics (shape, size, edge, elevation, form and opacity) on NA plates were recorded. Biochemical tests were performed for Gram positive bacteria, including oxidase (OX), catalase (CAT), Bile Esculin Agar (BEA) tests, and for Gram negative bacteria, including Kliger's Iron Agar (KIA), motility, indole urea (MIU), citrate utilization (CIT), carbohydrate fermentation (dextrose, sucrose, lactose) methyl red (MR), and Voges-proskauer (VP) tests [11].

Isolate antibiotic sensitivity assay

A standard agar-disk-diffusion method was used to perform antibiotic susceptibility assays using 21 commercially available antibiotic discs (Oxoid) that belong to 12 groups [12]. Mueller—Hinton agar (MHA) (Oxoid) media was used for the assay. Inhibition zone sizes were interpreted by referring to zone diameter interpretive standards of the National Committee for Clinical Laboratory Standards (NCCLS) 2000 (Supplementary materials Table S1).

Amplified ribosomal DNA restriction analysis (ARDRA)

Chromosomal DNA of each isolate was extracted from a fresh bacterial culture (<10^9 cells/mL) using an ATP™ Genomic DNA Mini Kit (ATP Biotech Inc., USA). PCR was performed with a G2 hot start master mix (Promega, USA) using universal primers 27F (5′-AGAGTTTGATCMTGG CTCAG-3′) and 1492R (5′-GGTTACCTTGTTAGGACTT-3′) for the bacterial 16S rRNA gene. The PCR reaction involved initial heating at 96 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min 30 s; and a final extension of 10 min at 72 °C. The PCR tubes were then stored at −20 °C for analysis. Restriction digestion of the 16S rRNA gene PCR product (approximately 1400 bp) from the isolates was performed using the HhaI enzyme (Bio labs, England). The reaction mixture contained 2 μl of 10× buffer, 0.2 μl of bovine serum albumin, 6U HhaI enzyme, 2.5 μl of water, and 1000 ng of PCR product. The digests were visualized after 1.5% (wt/vol) agarose gel electrophoresis. Different sizes of DNA markers (1 Kb and 100 bp; Bioneer, South Korea) were used to analyze the different restriction fragments.

Sequencing and phylogenetic analysis

The 16S *rRNA* gene PCR products of the isolates from each ARDRA group were purified using the Wizard PCR SV Gel and PCR Clean-Up System kit (Promega, USA) and sequenced (ABI Prism 3130 Genetic Analyzer, USA) using forward 27F and reverse 1492R primers. Partial sequences were combined via the SeqMan Genome Assembler (DNAstar, USA) and were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) using the Basic Local Alignment Search Tool (BLAST) to identify close phylogenetic relatives. Multiple sequence alignment of the retrieved reference sequences from NCBI was performed using ClustalW [13] software and exported to the Molecular Evolutionary Genetics Analysis (MEGA) [14] program for phylogenetic tree construction using the neighbor joining algorithm and selecting 1000 bootstrap replication.

Analysis of C-reactive protein in blood samples

A total of 45 serum samples (30 DFI, 10 non-DFI and five healthy controls) were selected randomly to measure CRP levels using *Cardio* Phase *hs*CRP Reagent (Siemens Health care Diagnostics products GmbH, Germany). The reagent consists of a suspension of polystyrene particles that had been coated with mouse monoclonal antibodies

(<0.016 g/L) to CRP (BN * II/BN prospect System). Polystyrene particles that had been coated with monoclonal antibodies specific for human CRP aggregate when mixed with samples containing CRP. These aggregates scatter a beam of light that is passed through the sample. The scattered light intensity is proportional to the relevant protein concentration in the sample. The results are evaluated by comparison with a standard of known concentration.

Statistical analysis

To assess the significance of occurrence of the isolates in the DFI and non-DFI groups, a z-test calculator for proportions was used to investigate whether the two populations differ significantly in proportion (http://www.socscistatistics.com/tests/ztest/Default2.aspx). A two-sample or independent sample t-test was used to compare the differences in TVBC, TEC, and TARC between DFI and non-DFI samples. The t-score to p-value transformation was performed using the Student's T cumulative distribution function (http://in-silico.net/tools/statistics/). The regression analysis was performed to examine correlations between CRP levels, and the number of antibiotic-resistant isolates in DFI and non-DFI ulcers was compared using the statistical program in Microsoft Excel; 2010, USA.

Results

Demographic data of diabetic and non-diabetic subjects

This study was conducted using 67 DFI patients and 12 non-DFI (control) patients. The patients were grouped according to their sex and age distribution, occupation, socio-economic status and lifestyle (Table 1). Most of the patients were from rural areas (middle income family) with an age range from 28 to 75 years and a mean age of 56.25 years. Males were the predominant study subjects. Occupationally, a majority of DFI patients were small-scale businessmen (39%) and housewives (21%). Among non-DFI patients, 91.6% were daily workers (Table 1).

Microbiological analyses of pus samples from diabetic (67 samples) and non-diabetic (12 samples) foot infections were performed to determine the total viable bacterial count (TVBC), the total *Enterobacteriaceae* count (TEC) and the total antibiotic resistant count (TARC) using the antibiotic supplements CIP (0.16 μg/ml) and CTX (1 μg/ml) or both antibiotics supplemented within NA and MAC media. The bacterial load (TVBC, TEC, TARC) in DFI was higher than that in non-DFI samples with a significance level of $p < 0.01$ (Fig. 1A).

A total of 213 bacteria were isolated from 67 DFI pus samples, whereas 131 bacteria were obtained from the 10 non-DFI specimens (two

Table 1 Demographic data representing sex, age, occupation, socioeconomic status of diabetic foot infection (DFI) and non-diabetic foot infection (non-DFI) study groups.

		DFI patients (67)	Non-DFI patients (12)
Sex	Male	47(70%)	11(91.6%)
	Female	20(30%)	1(8.33%)
Age	≤40 years	4 (6%)	9(75%)
	≥40 years	63(94%)	3(25%)
Occupation	Daily worker	5(7%)	11(91.6%)
	Business	26 (39%)	0
	House wife	14(21%)	1(8.3%)
	Farmer	7(10%)	0
	Service	4(6%)	0
	Service Rtd	8(12%)	0
	Teacher	3(4%)	0
Life style	Rural	47(70%)	11(91.6%)
	Urban	20(30%)	1(8.33%)
Socio-Economic Status	Low − income (BDT ≤5360)	9(13%)	1(8.33%)
	Lower middle-income (BDT 5361-21,270)	46(69%)	11(91.6%)
	Upper middle-income (BDT 21,271-65,671)	12(18%)	0
	High-income (BDT ≥65,672)	0	0

BDT, Currency of Bangladesh.

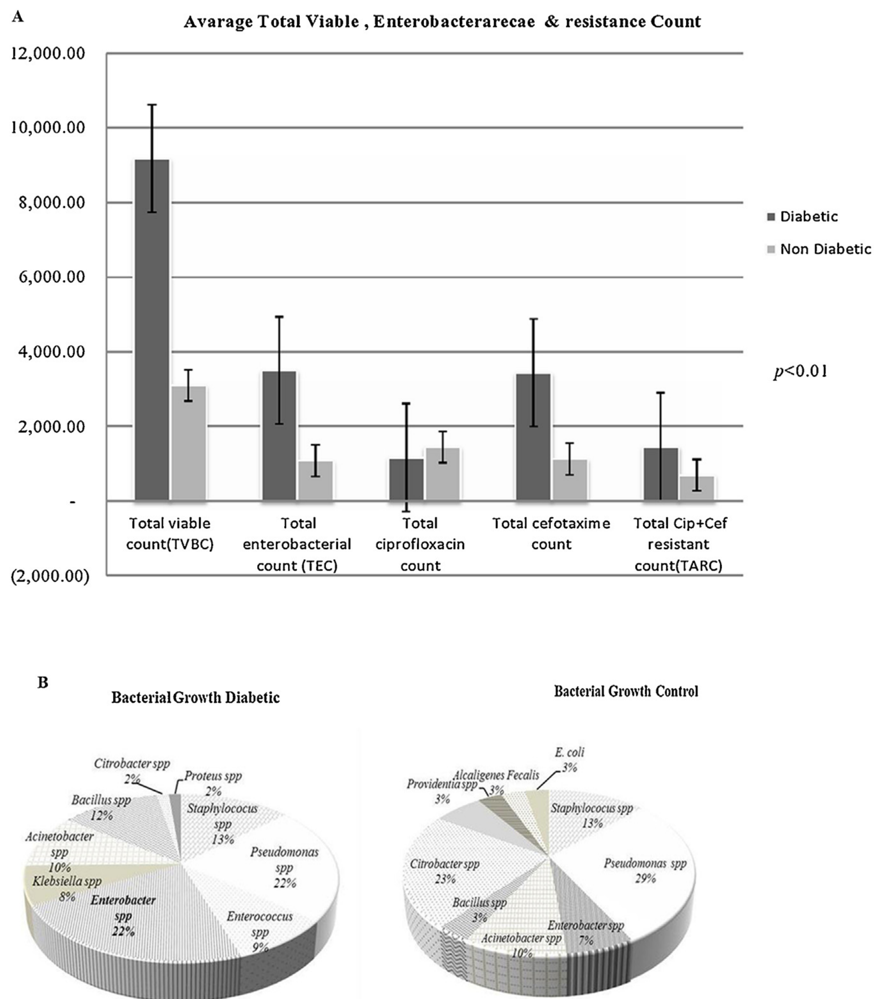

Figure 1 A: Total viable, *Enterobacteriaceae* and resistance bacterial count of pus samples from diabetic and non-diabetic foot infection patients. The error bars indicate the standard deviation. The standard deviation was calculated from the mean of duplicate results. B: Distribution of bacterial genera within pus samples of (a) diabetic and (b) non-diabetic foot infections. The presumptive genera were determined according to biochemical tests.

non-DFI specimens were bacterial culture negative). According to their phenotypes (colony morphology, gram staining, biochemical properties, and sugar fermentations), different morphological types were obtained. For population composition analysis, all of the isolates belonged to groups 1—10 (nine DFI and seven non-DFI isolates). The isolates were further extensively characterized up to the genus level following standard guidelines [11] using clustering software BioCluster 2.0 (www.Microbialgen.du.ac.bd/BioCluster). The organisms belonging to each morphogroup were as follows: group 1, *Staphylococcus* spp.; group 2, *Pseudomonas* spp.; group 3, *Enterococcus* spp.; group 4, *Enterobacter* spp.; group 5, *Klebsiella* spp.; group 6, *Acinetobacter* spp.; group 7, *Bacillus* spp.; group 8, *Citrobacter* spp.; group 9, *Proteus* spp.; and *Providentia* spp.; and group 10, *Alcaligenes* spp. (Fig. 1B, Supplementary materials Table S2).

Molecular characterization and genotyping: ARDRA, sequencing and phylogenetic analysis

ARDRA of the PCR products (approximately 1400 bp) of the different isolate morphogroups using the restriction enzyme *Hha*I revealed different restriction patterns, and the combined restriction fragments for each restricted amplicon were 1400 bp in total. ARDRA of 10 morphogroups demonstrated a distinct genotypic pattern except for morphogroup 2 (Supplementary materials Table S4), the isolates of which displayed five different patterns (Supplementary materials Fig. S1). From each of the

Table 2 Maximum identity profile of 16S rRNA gene sequences of isolates according to BLAST identification.

Morph groups	Isolates no	Accession numbers	Close similarity to (accession numbers)	% Maximum identity
Group1	39DMH3_Diabetic	(KM025375)	*Staphylococcus epidermidis*Y76 (JX067904)	99%
Group2	47DMAC2_Diabetic	(KM025388)	*Pseudomonas aeruginosa* C1501 (KF976394)	100%
	54DMH1_Diabetic	(KM025368)	*Pseudomonas stutzeri* TH-31 (KF783212)	100%
	45DCIP + CEF1_Diabetic	(KM025370)	*Pseudomonas monteilii* EU45 (JF681286)	100%
	48DMH1_Diabetic	(KM025369)	*Pseudomonas pseudoalcaligenes* Z170 (KF835816)	99%
	50DSWAB1_Diabetic	(KM025371)	*Pseudomonas hibiscicola* cp17 (JN082269)	100%
Group3	41DMH2_Diabetic	(KM025376)	*Enterococcus faecalis* CV7 (KF7224942)	100%
Group4	44DMH1_Diabetic	(KM025389)	*Enterobacter hormaechei* WW2 (JN993998)	100%
Group5	49DSWAB2_Diabetic	(KM025377)	*Klebsiella pneumoniae* zg2010 (JX435602)	100%
Group6	44DMH2_Diabetic	(KM025378)	*Acinetobacter baumannii* OIFC143 (JN668837)	100%
Group7	46DMH2_Diabetic	(KM025374)	*Bacillus cereus* HS3 (KF922378)	99%
	44DSWAB2_Diabetic	(KM025373)	*Bacillus circulans* I1 (FJ009417)	100%
Group8	2CMH3_Nondiabetic	(KM025386)	*Citrobacter sp.* R5-325 (JQ659727)	99%
	4CCIP1_Nondiabetic	(KM025381)	*Escherichia coli* c120 (JQ781608)	99%
Group9	5CMAC1_Nondiabetic	(KM025382)	*Providencia stuartii* S2SA-Sa (JQ828866)	99%
	56DMH1_Diabetic	(KM025372)	*Proteus mirabilis* FUA1263 (JN102554)	99%
Group10	3CCEFO3_Nondiabetic	(KM025390)	*Alcaligenes faecalis* SH184 (KC172063)	99%

10 morphogroups and corresponding ARDRA groups, 27 representative isolates were randomly selected for detailed 16S *rRNA* gene sequence analysis. Maximum identity profiles of the 16S rRNA gene sequences of the isolates according to BLAST identification are given in Table 2. The phylogenetic trees of gram-positive and gram-negative isolates are presented in Figs. 2 and 3, respectively.

Antibiogram of the isolated bacterial populations

An antibiogram was performed of DFI and non-DFI isolates using 21 commonly used antibiotics belonging to 12 different groups (Table S1). Bacteria isolated from DFI samples were more resistant to most antibiotics than bacteria that had been isolated from non-DFI samples. *Staphylococcus* spp. that had been isolated from DFI patients were 100% resistant to the monobactam (ATM) group of antibiotics and 67% resistant to the penicillin-G (PEN) group of antibiotics. However, non-DFI isolates were 100% sensitive to penicillin. *Pseudomonas* spp. DFI isolates were 33%, 72% and 78% resistant to the carbapenem (IPM), cephalosporin (CAZ, CRO, CXM) and monobactam (ATM) groups of antibiotics, respectively, in contrast to non-DFI isolates, for which these values were 8%, 62%, and 69%, respectively. *Enterobacter* spp. isolated from both DFI and non-DFI patients demonstrated a similar pattern of resistance to penicillin (PEN), monobactam (ATM) and cephalosporin (CAZ, CRO, CXM) antibiotic groups. *Acinetobacter* spp. isolated from DFI were 86% resistant to penicillin (PEN) and cephalosporin (CAZ, CRO, CXM) antibiotic groups in contrast to non-DFI isolates, which were 57% resistant. *Bacillus* spp. isolated from DFI and non-DFI were, respectively, 88% and 50% resistant to ATM, cephalosporin (CAZ, CRO, CXM), and penicillin (PEN) antibiotic groups. *Citrobacter* spp. that were isolated from both DFI and non-DFI patients were 100% resistant to the cephalosporin antibiotic group. However, on average, 82% of DFI isolates and 90% of non-DFI isolates were sensitive to the carbapenem (IPM) antibiotic group.

Analysis of C-reactive protein

The normal range of CRP levels in human serum is $\leq$10 mg/L [15]. In DFI patients, serum CRP levels ranged from 11.1 to 201 mg/L (mean value 53.80 ± 49.93 mg/L); however, in non-DFI patients, the levels ranged from 9.1 to 96.5 mg/L (mean value 47.73 ± 37.84 mg/L), and in healthy controls, the levels were 4—6.25 mg/L (Fig. 4A).

The CRP values increased with chronic DFI and non-DFI patient history and concomitantly changed the microbe composition in our samples. We therefore investigated the relationship among the composition of the bacterial population, the resistance pattern and CRP values. The findings revealed the following pattern (Table 1) — (i) DFI patients with CRP values between 10 and 50 mg/L were commonly infected with *Pseudomonas* spp.,

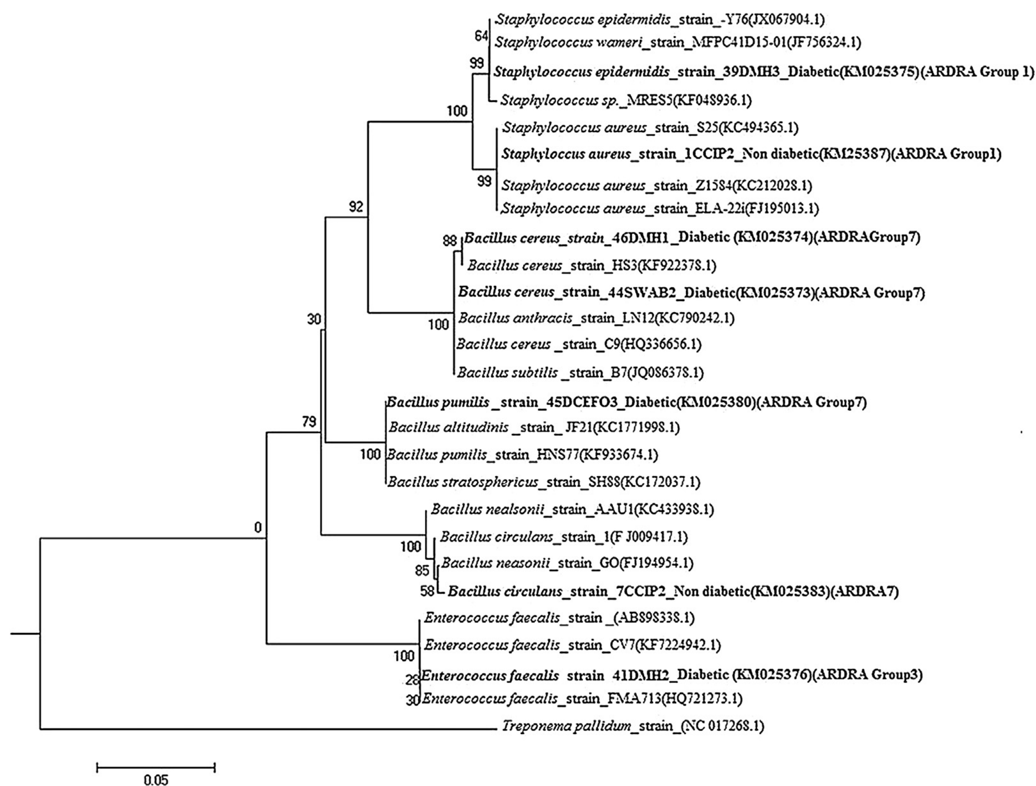

Figure 2 Phylogenetic tree of 16S rRNA gene sequences of Gram-positive isolates from diabetic and non-diabetic patient foot infections (Accession number shown in bold). The tree was generated in the MEGA 4 program using the neighbor-joining algorithm; the *Treponema pallidum* sequences served as the out group. Bootstrap values ($n = 1000$ replicates) are shown at branch nodes, and the scale bar represents the number of changes per nucleotide position.

Enterobacter spp. and *Klebsiella* spp. and were resistant to seven groups of antibiotics. In contrast, non-DFI patients with CRP values between 10 and 50 mg/L were commonly infected with *Acinetobacter* spp. and were resistant to six antibiotic groups; (ii) DFI patients with CRP values between 50 and 100 mg/L were commonly infected with *Enterobacter* spp., *Klebsiella* spp. and *Pseudomonas* spp. and were resistant to nine antibiotic groups. Non-DFI patients with CRP values between 50 and 100 mg/L were commonly infected with *Pseudomonas* spp., *Citrobacter* spp., and *Staphylococcus* spp. and were resistant to eight antibiotic groups; and (iii) DFI patients with CRP values >100 mg/L were commonly infected with *Bacillus* spp. and *Pseudomonas* spp. and were resistant to more than 12 groups of antibiotics. Data regression analysis revealed a correlation in DFI samples with $r = 0.656$, $p < 0.01$ and non-DFI $r = 0.0387$, $p < 0.26$ (Fig. 4B) (Table 3).

Discussion

The pathophysiological and metabolic differences in DFI and non-DFI patients indicates that there may be a difference in bacterial population compositions. To address this hypothesis, in this investigation, we analyzed bacteria in pus samples from 67 DFI and 12 non-DFI patients. The small number of non-DFI patient samples compared with DFI-patient samples in this investigation was due to low abundance of non-DFI patients compared with DFI patients. In our study, we determined that diabetic patients older than 40 years of age were more susceptible to foot infection, whereas this value for non-DFI patients was less than 40 years of age. The average age of DFI-patients was 58 years, and these patients were mostly male. Our study revealed that most of the DFI patients were from rural areas (lower middle income family), and the majority of them were using tube well water. A majority (39%) of DFI patients were small-scale businessmen, and 21% were housewives. However, 91.6% of the non-DFI patients were daily workers.

Approximately, 93.3% and 83.3% of DFI and non-DFI pus specimens, respectively, displayed culture-positive results; however, the bacterial population compositions were distinctly different in DFI patient pus samples compared to the

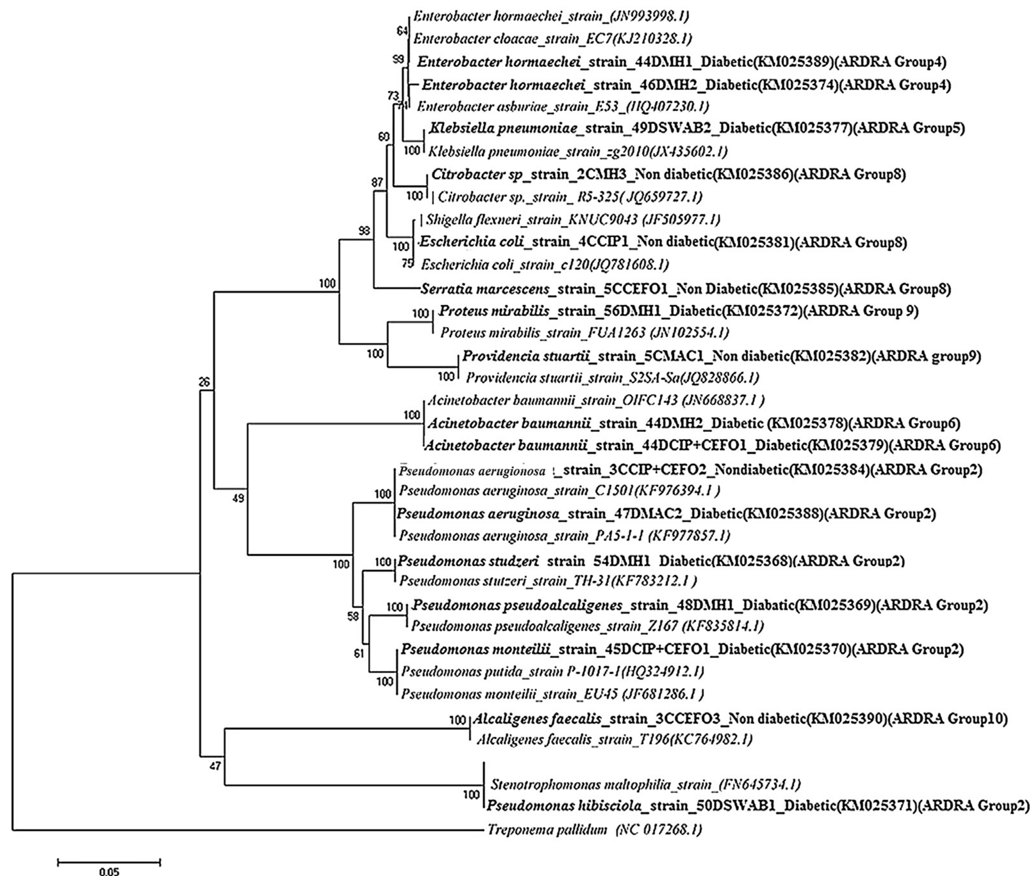

Figure 3 Phylogenetic tree of 16S rRNA gene sequences of Gram-negative isolates from diabetic and non-diabetic patient foot infections (Accession number shown in bold). The tree was generated in the MEGA 4 program using the neighbor-joining algorithm; the *Treponema pallidum* sequences served as an out group. Bootstrap values ($n = 1000$ replicates) are shown at branch nodes, and the scale bar represents the number of changes per nucleotide position.

samples from non-DFI patients. Among the isolates, gram-negative organisms were predominant in both patients. These results are compatible with the findings of previous studies [16,17]. However, a recent study found a predominance of Gram-positive organisms in non-DFI patients [18]. Our study demonstrated that most DFI and non-DFI patient infections were polymicrobial in nature. The statistical calculation (comparative bacterial significance analysis at 90% confidence level) revealed that the rate of occurrence of three organisms, namely, *Enterococcus* spp.

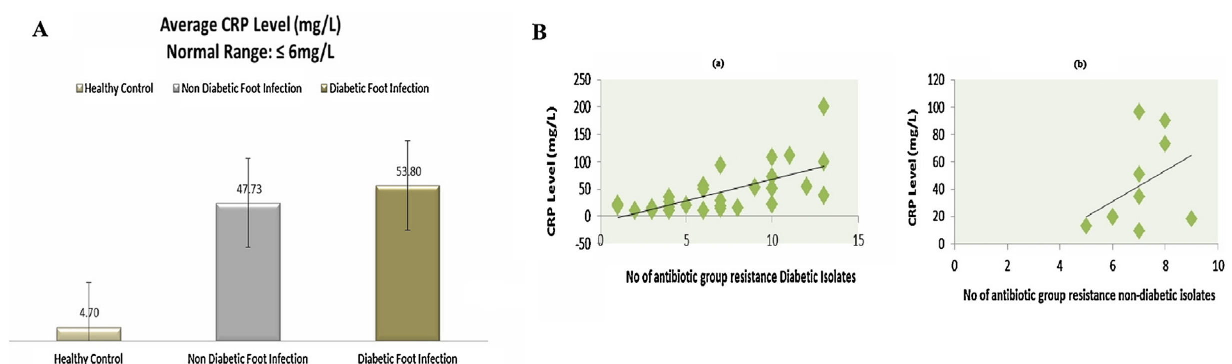

Figure 4 A: Average serum C reactive protein levels in diabetic and non-diabetic patients. B: Correlation between serum CRP levels and no antibiotic resistance by (a) diabetic and (b) non-diabetic isolates.

Table 3 Correlation between serum CRP levels, organism growth and degree of antibiotic resistance. (a) Diabetic patients and (b) non-diabetic patients.

CRP value range (mg/L)	Common organism growth	No of antibiotics commonly resistance	Groups
(a) Correlation with DFI isolate resistance pattern and CRP levels			
10—50	*Pseudomonas* spp. *Enterobacter* spp. *Klebsiella* spp.	AMC, ATM, AMP,CAZ,CRO, CXM,CFM,CN,DO (9)	7
50—100	*Pseudomonas* spp. *Enterobacter* spp.	AMC, ATM, AMP, CRO, CXM, CTX, CN, IPM,PB,DO (10)	8
>100	*Bacillus* spp. *Pseudomonas* spp.	AK,AMC,ATM,AMP,AZM,CAZ,CRO,CXM, CIP,C,CTX,IPM,CFMF,CO,DA,RD,PB (18)	12
(b) Correlation with non-DFI isolate resistance patterns and CRP levels			
10—50	*Pseudomonas* spp.	AMC, ATM, AMP, AZM,CAZ,CRO, CXM,CTX,CFM (9)	6
50—100	*Pseudomonas* spp. *Citrobacter* spp. *Staphylococcus* spp.	AMC, AMP, AZM,CAZCRO,CXM, CIP,C,CTX,DO,CO,DA (11)	8

$|Z| = 2.2125$, *Klebsiella* spp. $|Z| = 1.732$, and *Bacillus* spp. $|Z| = 1.9034$, were significant in DFI patients compared with non-DFI patients. However, in non-DFI patients, the rate of occurrence of *Citrobacter* spp. $|Z| = 14.1901$ was significant. Common genera found in both DFI and non-DFI include *Pseudomonas* spp., *Staphylococcus* spp. and *Acinetobacter* spp. These polymicrobial infections are responsible for chronic wounds and more complex infections.

Through phenotypic and genotypic characterization, it was observed that there were five strains of *Pseudomonas* spp. obtained from DFI, including *P. aeruginosa*, *P. stutzeri*, *P. pseudoalcaligens*, *P. monteilii*, and *P. hibiscicola* (Fig. 3). In this study, *Ent. hormaechei* were the predominant organism (22%) in DFI isolates and occurred in low (6%) numbers in non-DFI isolates. *Citrobacter* spp. are opportunistic nosocomial pathogens that cause 5% of infections in immune-compromised patients [16]. In our study, the prevalence of *Citrobacter* spp. was 33% in non-DFI; however, the prevalence was only 2% in DFI. The prevalence of *B. cereus* was 17% in DFI and 3% in non-DFI patients. In our study, *Enterococci* spp. (9%) and *Klebsiella* spp. (7%) were found in DFI pus samples, whereas these values were reported to be 14.9% and 20.5%, respectively, elsewhere [19]. *Staphylococcus* species comprised 13% of isolates in both DFI and non-DFI patients. *A. baumannii* is an important nosocomial pathogen [20]. In our study, *A. baumannii* demonstrated a similar percentage (10%) in both DFI and non-DFI patients. However, in many studies, 33% of *A. baumannii* strains were isolated from DFI patients [21,22].

DFI patients have chronic non-healing ulcers due to several underlying factors such as poor glycemic control, peripheral neuropathy, poor blood supply to the extremities and polymicrobial infections in their feet. These factors lead to CSI and delay the healing process [4,9,23]. Different pathogen types are associated with different inflammatory responses, and patients who were infected with Gram-negative organisms have high CRP levels. DFI patients with CSI are always treated (long term) with a combination of antibiotics because the microbes are highly resistant to different antibiotic groups [24,25]. In the present investigation, the bacterial population associated with DFI was comparatively more resistant to antibiotics than were the non-DFI isolates. CRP values in DFI patients were positively correlated with the bacterial population and antibiotic resistance properties of the isolated bacteria from DFI patients in our study. There was a predominance of Gram-negative organisms in pus samples from patients with high CRP levels. We observed that DFI patients who were commonly infected with *Pseudomonas* spp. and *Bacillus* spp. had high CRP levels. This result is compatible with findings from others [26]. *Bacillus* spp. and *Pseudomonas* spp. that were isolated from DFI patients were resistant to more than 12 groups of antibiotics. Non-DFI patients who were infected with *Pseudomonas* spp. and *Staphylococcus* spp. had high CRP levels. *Pseudomonas* spp. *and Staphylococcus* spp. that were isolated from non-DFI patients were commonly resistant to eight groups of antibiotics.

Our study revealed a significant correlation between MDR organism growth in foot infections and high serum CRP levels. We observed that patients infected with MDR microbes had high serum CRP levels. These findings are comparable with other studies [9,27]. Consequently, we can assume that the growth of Gram-negative MDR organisms in patients' foot infections influences serum CRP levels.

Conclusions

This investigation demonstrates a complex association of the bacterial population in DFI patients compared with non-DFI patients with different antimicrobial resistance patterns, which are linked with CRP levels. An altered microbial composition was observed in DFI and non-DFI patients. The microbiome associated with DFI and non-DFI patients demonstrate MDR properties at an alarming rate. This investigation also explores the correlation of serum CRP levels with microbial compositions and their resistance properties. This study is expected to generate valuable information, which will be helpful in the management and prevention of foot infections in our population and will help clinicians to select and develop appropriate drugs.

Ethical approval

All of the subjects (patients) were aware of their involvement in this study and gave their consent to participate in this research. This study was reviewed by the ethics committee of the Diabetic Association of Bangladesh (BADAS) **(reference number BADAS-ERC/EC/14/00137) (Supplementary materials)**.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jiph.2015.10.011.

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