Opportunistic Microorganisms Identification Using a Modern Laboratory Technique

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Abstract

Introduction: Recent studies show that 85% of cases of non-traumatic amputations in patients with diabetes mellitus are preceded by a wound infection of the foot, this confirms the need for rational antimicrobial therapy. Therefore, competent rapid identification of microorganisms is a priority task of modern microbiology. Matrix-Assisted Laser Desorption Ionization TOF-stands for Time of Flight Mass Spectrometry (MALDI-TOF MS) is considered as the revolution in bacteriology, to identify pathogens isolated in a clinical microbiological laboratory. In this regard, MALDI-TOF MS is an ideal solution for the rapid identification of DFS

Objectives: To Explore of Modern Laboratory Technique (MALDI-TOF MS) for the identification of opportunistic microorganisms in patients with Diabetic Foot Syndrome (DFS).

Materials and Methods: Was performed from September 2012 to May 2015 on patients admitted to the Emergency Room of the Hospital General de México. 72 patients with (DFS) diabetic foot syndrome were investigated for the period of 2013-2015 years. The comparison group consisted of 30 patients with chronic purulent inflammatory diseases of the lower extremities not suffering from diabetes mellitus. Identification of microorganisms was carried out using MALDI-TOF spectrometry (BRUKER 2012).

Results: This study revealed the prevalence gram-positive bacteria in patients with DFS. The dominant microorganism in the etiology of DFS was *S. aureus*, which occurred mostly as monoculture. Coagulase-Negative Staphylococci (CoNS) were represented mainly by *S. haemolyticus* and *S. epidermidis* generally recognized as a pathogen of medical devices in our case does not have an important role. Enterobacteria and non-fermenting bacteria represented Gram-negative bacteria**.**

Keywords: Coagulase -Negative Staphylococci; Diabetic Foot Syndrome; MALDI-TOF Spectrometry; *S. aureus*

Introduction

Patients with diabetes every year is steadily increasing. The following facts are given in WHO factsheets: the number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014.Diabetes is a major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation. In 2015, an estimated 1.6 million deaths were directly caused by diabetes. Another 2.2 million deaths were attributable to high blood glucose in 2012 [1]. One of the most common complications of diabetes is DFS - the gangrene of the foot develops in patients with diabetes mellitus, which is the main cause of limb amputation in more than 50% of patients, postoperative mortality rate is 13-20% [2]. Recent studies show that 85% of cases of non-traumatic amputations in patients with diabetes mellitus are preceded by a wound infection of the foot, this confirms the need for rational

antimicrobial therapy [3]. Therefore, competent rapid identification of microorganisms is a priority task of modern microbiology. Currently microorganisms are best identified using 16S rRNA and 18S rRNA gene sequencing. However, in recent years' matrix assisted laser desorption ionization-time of flight mass spectrometry has emerged as a potential tool for microbial identification and diagnosis Bizzini A et al. [4,5]. It is believed that MALDI-TOF mass spectrometry can be used as an alternative to sequencing the 16S rRNA gene to identify a pathogen [6]. Numerous studies confirm that MALDI-TOF MS equivalent or even surpassed the traditional diagnostic methods in speed and accuracy in detecting blood stream infections [7,8]. MALDI-TOF MS is considered as the revolution in bacteriology, to identify pathogens isolated in a clinical microbiological laboratory [9,10]. Traditional culture method is a costly and time consuming process requiring 3-5 days for detection and identification of the pathogens. Numerous studies have shown that MALDI-TOF MS is a rapid, reliable and cost-effective technique for identification of bacteria [11]. In this regard, MALDI-TOF MS is an ideal solution for the rapid identification of DFS pathogens to carry out competent antimicrobial therapy.

Materials and Methods

The sample consisted of 72 patients admitted to the Emergency Room of the Karaganda city hospital number 1" and "Regional Medical Center from September 2013 to May 2015 years. The patients were diagnosed with type 2 Diabetes Mellitus (T2DM). and infected diabetic foot ulcers. The comparison group consisted of 30 patients with chronic purulent-inflammatory diseases of the lower extremities, with no history of diabetes mellitus. Surgeons assessed the ulcers, sample for culture was collected with a cotton-tipped sterile swab from the deeper parts of the foot ulcer. Initially, wound contents were examined by a microscopic method. An approximate identification of microorganisms was carried out in Gram-stained smears. Swabs received were also cultured on mannitol salt agar, meat-peptone agar, blood agar, Sabouraud gentamicin-chloramphenicol agar and the plates were incubated overnight at 37 °C in aerobic conditions. Anaerobic incubation was not carried out due to lack of equipment. Colonies obtained were identified by direct application method using standard operational protocol on MALDI-TOF spectrometry (Bruker Daltonics 2012). The biomaterial (single colony) was distributed in a thin layer directly to the target point on the metal plate, starting from the middle. Cover the dots with the applied biomaterial 1 µl of the α-cyano-4-Hydroxycinnamic Acid (CHCA) matrix solution for an hour after drying and allow to dry completely at room temperature. After the crystallization of the matrix and compound, the target on the metal plate is introduced in the mass spectrometer. Bioanalytes separated according to their TOF create a mass spectrum. A spectrum is thus a microbial signature that is compared with a database for the identification at the species or genus level [12].

Statistical Analysis was performed using the STATISTICA-6 package.

The relative frequency (p) of the occurrence of an attribute was

determined as follows: $p = \dfrac{k}{n}$,

k - Number of cases with the attribute of interest

n - Sample size

The attribute is defined as a specific characteristic or feature of a given subject

p is calculated by sample; it reflects the population with some error:

$$m_p = \sqrt{\frac{p \times (1-p)}{n}}$$

The confidence interval for the p is located within

$$p - t_a \times \sqrt{\frac{p(1-p)}{n}} \quad \text{and} \quad p + t_a \times \sqrt{\frac{p(1-p)}{n}}$$

tα- is the critical value of the bilateral t-criterion of the Student for a given α and (n1 + n2-2) degrees of freedom. To compare the relative frequency of occurrence of an attribute in different independent sets, the criterion z was used:

$$z = \frac{p_1 - p_2}{\sqrt{m_{p1}^2 + m_{p2}^2}}$$

Differences were considered statistically significant at p < 0.05

Results

Results of the 72 patients with DFS enrolled for the study, 19 (26.39%) were males and 53 (73.61%) were females, with a male to female ratio of 1: 2.8. The age range was 40-77 years with mean age 54 ± 7.8 years. Of the 30 patients comparison group 14 (46.66%) were males and 16 (53.34%) were females. The age range was 28-60 years with mean age 44 ± 9.1 years. A total of 100 isolates were obtained from the 72 patients with DFS, accounting for the average of 1.39 isolates per subject. Mono-microbial infection occurred among 46 (64%) patients with DFS while polymicrobial infection occurred among 28 (36%). Figure 1 shows the distribution of bacterial isolates among patients with DFS.
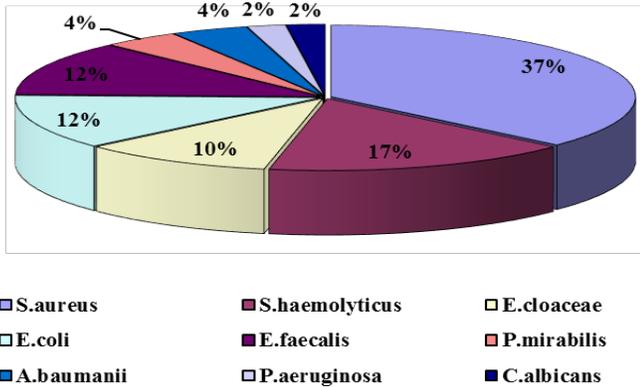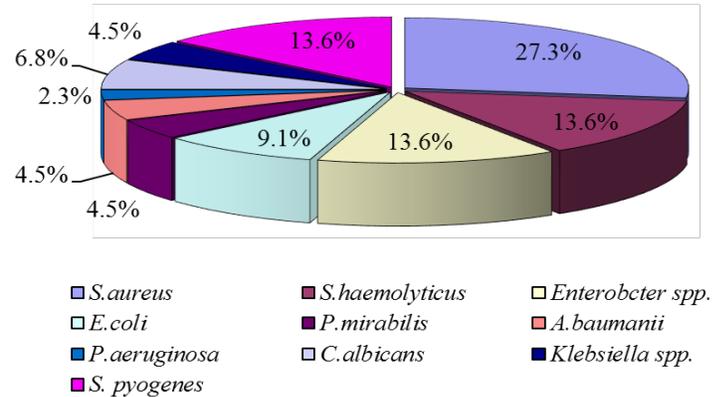
Figure 1: Distribution of bacterial isolates among patients with DFS.

S. aureus had the highest degree of occurrence (37%) followed by *S. haemolyticus* (17%). *The proportion of E. coli, E.faecalis and E.cloaceae was (12%), (12%) and (10%) respectively. P.mirabilis (4%) A.baumanii (4%), P.aeruginosa (2%) C.albicans (2%) were also isolated.*

S. aureus and *P. aeruginosa* were isolated as monomicrobial culture; at the same time *P. mirabilis, A. baumanii,C. albicans* occurred in mix culture, the most frequent combination with Coagulase -Negative Staphylococci (CoNS). A total of 44 isolates were obtained from the 30 comparison group patients accounting for the average of 1.47 isolates per subject. Monomicrobial infection occurred among 22 (73.3%)) patients while polymicrobial infection occurred among 8 (26.7%). Figure 2 shows the distribution of bacterial isolates among comparison group patients.

Figure 2: Distribution of bacterial isolates among comparison group patients.

S. aureus had the highest degree of occurrence (27.3%) followed by *S. haemolyticus, S.pyogenes* and *Enterobacter spp.* (13.6%).

There was a statistically significant difference between gram-positive and gram-negative bacteria in patients with DFS (Table 1), in the comparison group there was only a slight predominance of gram-positive microorganisms over gram-negative.

Microorganisms	DFS patients					Comparison group patients			
	n	p%	m_p	95% CI	Critical t-value (α) <	n	p%	m_p	95%CI
Gram-positive bacteria	66	66	4.73	9.28		24	54.54	4.97	10.03
Gram-negative bacteria	32	32	4.66	9.14	0.005	16	38.64	7.34	14.7
Fungi (Candida)	2	2	1.4	2.74		3	6.82	3.8	7.67
Total	100	100				43	100		

Table 1: Comparison of isolates of Gram-positive and Gram-negative bacteria by subjects.

Isolates of *S. aureus* are statistically significant higher in patients with DFS (Table 2), compared with *S. haemolyticus* and *E. faecalis* (α <0.01 and 0.005)

Microorganisms	DFS patients				Comparison group patients			
	(n) p%	m_p	Critical t-value (α)	95% CI	n	p%	m_p	95% CI
Gram-positive bacteria								
S. aureus	37	4.83		9.46	12	27.3	4.57	9.24
S. haemolyticus	17	3.76	<0.01	7.06	6	13.6	3.43	6.92
E. faecalis	12	3.25	<0.005	6.37	-	-	-	-
S.pyogenes	-	-		-	6	13.6	3.43	6.92
Gram-negative bacteria								
E. coli	12	3.25		6.37	4	9.1	5,3	10.7
Enterobacter spp.	10	3.0	*	5.88	6	13.6	5,17	10.45
Klebsiella spp.	-	-	*	-	2	4.54	3,13	6.32
P. mirabilis	4	1.96	*	3.84	2	4.54	3,13	6.32
A.baumanii	4	1.96	*	3.84	2	4.54	3,13	6.32
Pseudomonas	2	1.4		2.74	1	2.3	2.25	4.51
Candida	2	1.4		2.74	3	6.84	3.8	7.67
Total	100				44	100		
* -α >0,05								

Table 2: Comparison of isolates by subjects.

S.pyogenes was isolated only in comparison group patients. Among the gram-negative bacteria *E. coli* and *Enterobacter* spp. dominated in both groups. *P. mirabilis, A baumanii, Pseudomonas* and Candida were isolated in insignificant amounts. *Klebsiella* were isolated from patients of the comparison group

Discussion

The annual population-based incidence of diabetic foot ulcers is estimated to be 1.0-4.1 %, while the lifetime rate extends to around 25 % [13] A common complication of these ulcers is infection, which if left untreated, results in the need for distal limb amputation [14]. In present study 53 (73,61%) females and 19 (26.39%) were males, with a male to female ratio of 1:2.8. This is significantly different from most study results in which have reported male sex as a significant risk factor for non-healing ulcer [15]. The age range was 40-77 years with mean age 54 ± 7.8 years. A total of 100 aerobic and facultative anaerobic bacterial isolates were encountered in the 72 patients with DFS accounting for an average of 1.39 isolates per subject. It is it is no different from comparison group patients accounting for the average of 1.47 isolates per subject. Mono-microbial infection occurred among 46 (64%) patients with DFS while polymicrobial infection occurred

among 28 (36%). *S. aureus* and *P. aeruginosa* were isolated as mono-microbial culture; at the same time *P. mirabilis*, *A. baumanii*, *C. albicans* occurred in mix culture, the most frequent combination with coagulase -negative staphylococci (CoNS).

We found that the infection in most diabetic foot lesions was polymicrobial. This pathogen isolation rate per lesion is 1.97 reported by Sarita Otta et al. [16] and 2.37 reported by Carvalho et al. [17] The predominance 75.6 per cent of gram-negative organisms has been noted in several studies [18]. However, certain studies [19,20] have established a higher proportion of gram-positive organisms. In this study, 66% gram-positive and 34% gram-negative bacteria isolated from patient with DFS. *S. aureus* was the most commonly isolated organisms (37 %) followed by S. haemolyticus (17%). CoNS were represented mainly by *S. haemolyticus*. *S. epidermidis*, generally recognized as a pathogen of medical devices, in our case does not have an important role. *S.pyogenes* was isolated only in comparison group patients. Among the gram-negative bacteria *E. coli* and *Enterobacter* spp. dominated in both groups. Ramakant et al. [21] in Lucknow, India, reported P. aeruginosa as the most common gram-negative isolate in diabetic foot ulcer patients in their study. Similar results were obtained by Ofonime M. Ogba et al. Pseudomonas aeruginosa was the most common gram-negative isolate to 24.7% in patient with DFS. In present studies Pseudomonas aeruginosa like *P. mirabilis, A. baumanii*, and Candida were isolated in insignificant amounts. Klebsiella were only isolated from patients of the comparison group

Conclusion

MALDI-TOF MS is a rapid, reliable and cost-effective technique for identification of bacteria in patient with DFS. A total of 100 aerobic and facultative anaerobic bacterial isolates were encountered in the 72 patients with DFS accounting for an average of 1.39 isolates per subject. Mono-microbial infection occurred among 46 (64%) patients with DFS while polymicrobial infection occurred among 28 (36%). Gram positive microorganisms prevailed over Gram -negative 66% and 34% respectively. The dominant microorganism in the etiology of DFS was *S.aureus* (37%), which occurred mostly as monoculture. CoNS were represented mainly by *S. haemolyticus*. *S. epidermidis*, generally recognized as a pathogen of medical devices, in our case does not have an important role. Among the gram-negative bacteria *E. coli* and *Enterobacter* spp. dominated in both groups.

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