ORIGINAL ARTICLE

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) for the Identification of Bacteria Causing Urinary Tract Infections

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ABSTRACT

Background: *Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) technology is a technique which allows microbial identification in clinical microbiology laboratories based on microbial protein profiles. A promising application of the MALDI-TOF technique comprises carrying out identification from direct urine samples.* ***Objectives:*** *The aim of this study is to evaluate the performance of MALDI-TOF MS system for the rapid identification of clinically relevant urinary tract pathogens in urine samples to the species level.* ***Methodology:*** *two hundred (200) urine samples with findings suggestive of urinary tract infections (UTI) were identified directly by MALDI-TOF MS, and same samples were identified from culture on MALDI-TOF MS. 73 colonies from 73 samples were further identified by Bd Phoneix and PCR.* ***Results:*** *MALDI-TOF MS directly from samples identified 174 samples (91 to the species level 83 to genus level), while MALDI-TOF MS after culture identified 191 samples (187 to the level of species, and 4 to genus level). In addition, 9 samples gave no results from the direct method and no growth in culture. The sensitivity and specificity of direct method compared to after culture method was 91.1% and 100%, respectively. Colonies of 73 samples were detected by the BD Phoneix automated identification system and by PCR. The results were identical among the three systems.* ***Conclusion:*** *The ability of MALDI-TOF MS's to detect microorganisms from urine samples directly can provide early diagnosis of UTIs and avoid the inadequate or unnecessary empirical antimicrobial treatment.*

INTRODUCTION

UTIs are a serious public health issue caused by a variety of bacteria. but most commonly by *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus saprophyticus*. Due to the increase in recurrence rates and rising antimicrobial resistance among uropathogens, these pose a serious threat to the financial burden of these illnesses [1].

UTI takes about 24 to 48 hours to be confirmed microbiologically. In the meanwhile, patients are always treated with empirical antibiotic treatment, which is often unnecessary or ineffective. Anticipation of clinically useful and valuable information is very critical and with supreme importance, with both diagnostic and therapeutic consequences[2].

Conventional microbiological techniques as biochemical and phenotypic analysis; using both manual and automated systems, in addition to molecular methods are always used in bacterial identification. While some of these techniques are rapid, the majority depend on microbial growth and utilization of biochemicals, requiring hours to days for identification[3].

Standardized test systems such as API and VITEKH 2 (bioMe´rieux), or PHOENIXH (BD Diagnostics), complemented by traditional culture and microscopy methods, have so far been used in routine labs for the rapid identification of clinical microorganisms. Using these techniques, the mean time required for a verified and validated identification ranged from 6 h to 18 h and in recent years. Analysis of selected genes by PCR methods or small-subunit rRNAs has complemented the biochemical tests, and reducing the time and providing the gold standard technique in several cases [4].

Conventional methods of bacterial species identification are time consuming and labor intensive. Gram stain and automated biochemical reaction reading systems such as Vitek 2 (bioMérieux, Marcy-l'Etoile, France) or BD Phoenix (Becton Dickinson, Sparks, MD), as well as antimicrobial susceptibility testing, still take 48–72 hours from sample collection to bacterial species identification.. MALDI-TOF MS has been created considerably in recent years and now constitutes

a quantum leap in the identification of pathogenic microbes. The technology allows for bacterial and fungal identification down to the genus and species level in a very quick, cost-effective, simple, and reliable manner [5].

On the other hand MALDI-TOF MS applications, can provide direct identification from clinical specimens like urine or cerebrospinal fluid making a significant impact in the treatment of patients with serious infections. Furthermore, the future potential has not been completely explored, and new applications are being developed, such as fast typing in outbreaks, early detection of antimicrobial resistance and identification of mixed infections employing advanced software algorithms [5,6].

Objective

The aim of this study was to evaluate the performance of MALDI-TOF MS system for the rapid identification of clinically relevant urinary tract pathogens in urine samples to the species level and to compare the results with the routine conventional methods for identification including UTI chromagar and the Phoenix.

To detect the sensitivity and specificity of MALDI-TOF MS for identification of bacterial isolates when compared to PCR as gold standard test.

METHODOLOGY

Cases:

Two hundred (200) patients with clinical manifestations suggested of UTIs presented to the Microbiology Laboratory at Central Lab of Armed Forces. Patients was subjected to full history taking.

Methods:

Two hundred (200 samples) of 10-20 ml midstream urine were collected from the patients in a sterile, dry, wide-necked, leak-proof container.

- Microbiological investigations:

Inclusion criteria to select positive sample were (positive dipstik test, WBCs > 10 HPF and direct film stained with Gram stain.

Positive samples were divided into 2 sterile containers.

When different colonies were found, the urine sample was rejected to exclude mixed infection. One of the 2 positive samples was processed for direct identification by MALDI-TOF. The second positive sample was inoculated onto CLED agar and UTi Chromagar plates. Plates were incubated in aerobic atmosphere at 37°C for 18-24 hr. When bacterial growth was observed, the colonies on chromagar were identified by morphology according to manufacturer. All isolates on CLED agar were identified by MALDI-TOF.

Seventy-three isolates were identified by BD Phoneix and polymerase chain reaction (PCR).

Identification of isolates:-

Conventional identification of Urine culture:

For the conventional urine culture, 1 µL of well-mixed urine was inoculated and spread onto blood agar plates and CLED agar plates using a sterile plastic disposable loop.

Plates were incubated in an aerobic atmosphere at 37℃ for 18-24 hr. When bacterial growth was observed, the colonies on CLED agar were counted, and identified morphologically.

MALDI-TOF mass spectrometry:

Procedure: The urine samples were processed as follows in order to acquire direct identification:

After homogenizing the samples with agitation, 5 mL of the mixture were centrifuged for 5 minutes at 700 rpm. The supernatant obtained was centrifuged for 15 min at 6000 rpm. The sediment was re-suspended in 1 ml of HPLC quality distilled water and was centrifuged again for 15 min at 6000 rpm. The proteins of the sediment were extracted using the ethanol/formic acid technique. The sample was combined with 300 litres of HPLC quality distilled water and 900 litres of 100% ethanol, then vortexed until a homogenous suspension was produced. The mixture was centrifuged at 13,000 rpm for 2 minutes, and the supernatant was removed. Finally, $25\,\mu L$ of 70% formic acid and $25\,\mu L$ of acetonitrile were added, vortexed and centrifuged for 1 min at 13,000 rpm. Using the supernatant obtained, four $1\ \mu L$ spots were dropped on four different areas of the metal sample plate (MSP 96 target polished steel; Bruker, Bremen, Germany). They were allowed to dry and $1\ \mu L$ of the matrix solution (saturated solution of cyano-4-hydroxy cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid) was added to each spot to be analyzed. They were allowed to dry again and then, a reading of the four spots from each sample was taken. All chemicals were supplied by Sigma–Aldrich (St Louis, MO, USA).

MALDI-TOF mass spectrometry measurements were performed on an Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Leipzig,Germany) equipped with a LTB MNL100-Hz Smart beam laser.

Data analysis: For automated data analysis, raw spectra were processed using the MALDI Biotyper Comass v4.3 software (Bruker Daltonics, Leipzig, Germany) at default settings

Result scoring. MALDI-TOF MS identifications were classified using modified score values proposed by the manufacturer:

When the results obtained by conventional microbiological methods and MALDI-TOF MS were inconsistent, both were repeated. According to the manufacturer, a score of_2.0 indicates species-level identification, a score between 1.7 and 1.99 indicates

genus-level identification, and a score of 1.7 indicates no identification.

However, we recorded the first ten identification options provided by the MALDI-TOF MS software, considering species-level prpper identification to be those options that agreed with the urine culture.

Phoenix:-

Seventy-three samples were confirmed by BD Phoenix (Becton-Dickinson company, USA), according to manufacturer's instructions.

Real Time PCR:-

Seventy-three samples were confirmed by real time PCR detection kit (DNA-Technology, Russia). Extraction was done by Prep-Na (DNA-Technology, Russia) on DT-PRIME instrument (DNA-Technology, Russia).

Statistical analysis:

Analysis of data was done by using SPSS (statistical program for social science version 25). The mean and standard deviation were used to represent the data. Frequency and percentage were used to express qualitative data. The relationship between qualitative factors was investigated using the Chi-square test. Fisher exact test was used instead of chi-square when one or more expected cell <5. For quantitative data, comparison between two groups y was done using student t-test or the corresponding non-parametric one (Mann-Whitney test) for variables not normally distributed. A p-value less than 0.05 was considered significant.

RESULTS

Two hundred patients from the Central Laboratory of Armed Forces were selected to participate in this study on the basis of clinical suspicious of UTI. They were recruited during the period from October 2016 to October 2017.They were 98 males and 102 females. Their age ranged from 15 to 77 years with mean age of (43.65 ±15.22 years). Regarding associated diseases 17% of patients with Diabetes mellitus and 22% of patients with Hypertension.

1- MALDI-TOF direct:

After processing urine samples for MALDI-TOF MS, 6 samples (3%) were insufficient pellet so these were not further studied, 9(4.5%) showed no peak and 11(5.5%) were unidentified. 174 samples (87%) were correctly identified. No microorganisms were misidentified (fig 1).

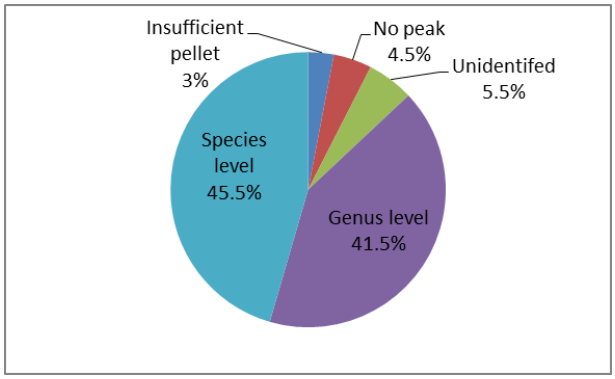

Fig 1: The percentage of identification by MALDI-TOF direct from urine samples.

Among insufficient pellet samples, 100% were Gram-negative bacteria. Among unidentified samples 100% were Gram-negative bacteria.

Table 1: Identified Organisms Directly by MALDI-TOF in comparison with Gram stain

	Gram stain	
MALDI Direct Organisms	Gram -ve Bacilli No. (%)	Gram +ve Cocci No. (%)
E. coli	96 (49.7)	0
K. pneumonia	39 (20.2)	0
P. aeroginosa	14 (7.3)	0
P mirabilis	6 (3.1)	0
A. baumanii	4 (2.1)	0
C. freundi	2 (1.0)	0
M. morgagni	1 (0.5)	0
E. cloacae	1 (0.5)	0
E. faecalis	0	4 (57.1)
Enterobacter	1 (0.5)	0
Pseudomonas	1 (0.5)	0
Serratia	1 (0.5)	0
S. aureus	0	3 (42.9)
Not Identified	26 (13.5)	0
Total	193	7

Table 1 shows that Gram negative microorganisms were 193 (96.5%) samples out of 200 and gram-positive bacteria were 7 (3.5%) cases out of 200.

Table 2: MALDI Direct Score in Identified and Unidentified Specimen

	MALDI Direct	**N**	**Mean**	**S.D**
Score	Identified	174	2.00011	.171760
	Unidentified	11	1.51182	.160861
	t-test	9.175	P value	<0.001*

The table shows that MALDI-TOF identification direct from urine samples was highly significant in identified samples score.

Table 3: Frequency and percent of organisms identified by direct MALDI-TOF MS

	Frequency	Percent%
Unidentified	11	5.5
Insufficient pellet	6	3.0
no peak	9	4.5
Genus Level Only		
E. coli	43	21.5
K. pneumoniae	17	8.5
P. aeruginosa	9	4.5
P. mirabilis	3	1.5
A. baumannii	2	1.0
E. faecalis	2	1.0
S. auerus	3	1.5
C. Freundi	2	1.0
E. cloacae	2	1.0
M. morgagani	0	0
Species level		
E. coli	53	26.5
K. pneumoniae	22	11
P. aeruginosa	6	3
P. mirabilis	3	1.5
A. baumannii	2	1.0
E. faecalis	2	1.0
S. auerus	1	0.5
C. Freundi	0	0.0
E. cloacae	0	0.0
S. marcescens	1	0.5
M. morgagani	1	0.5
Total	200	100.0

The table shows that among correctly identified samples 174 (87%) (of urine) correctly identified 91 (45.5%) showed a species level identification score ≥1.99, 83 (41.5%) showed a genus level identification score >1.7 and <1.99.

The majority of correctly identified samples were Gram-negative bacteria, 95.8%, versus 4.2% of Gram-positive bacteria. The most commonly isolated microorganisms were *Escherichia coli* 96 (56%), *Klebsiella pneumoniae* 39 (21.4%) and *Pseudomonas aeruginosa* 15(9.4%) (fig 2).

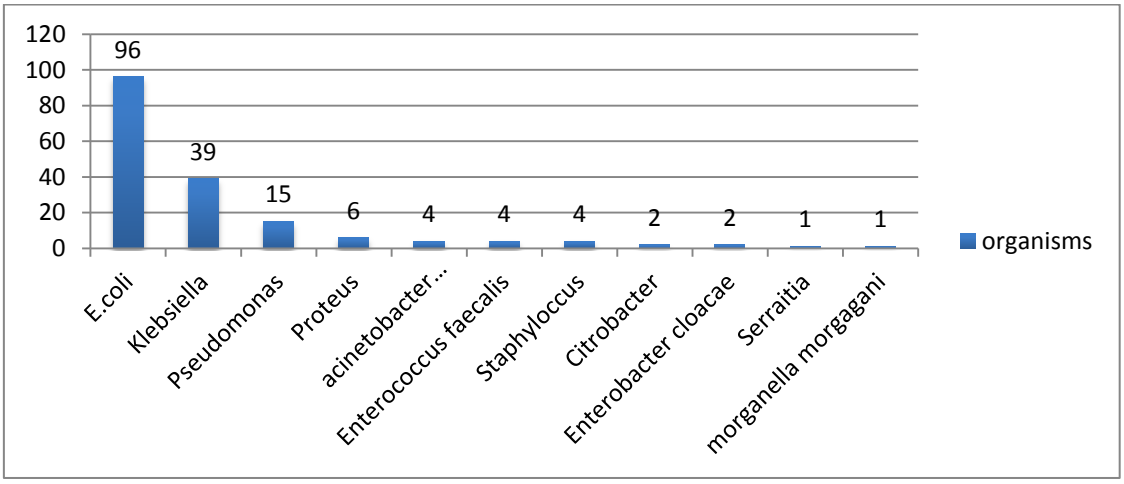

Fig 2: The different spp. isolated by direct method.

Table 4: Different groups of identification by direct method in relation to score.

	N	Mini.	Maxi.	Mean	Std. Deviation	F	Sig.
Not Identified (direct)	11	1.17	1.68	1.5118	.16086	278.475	0.0001 HS
Genus level (direct)	83	1.70	1.99	1.853	.09447		
Species level (direct)	91	2.00	2.44	2.1343	.10208		
Total	185			1.9711	.20628		

Test used one way anova P sig if ≤ 0.05.

The table shows that identification by MALDI-TOF directly from urine samples was highly significant in different groups in relation to score of identification.

MALDI-TOF identification after cultures: -

Out of 200 urine cultures,9 samples (4.5%) yielded no growth, 164 samples (82%) yielded colony counts equal to or greater than 10^5 CFU/ml, 27 (13.5%) lower than 10^5 CFU/ml (fig 3).

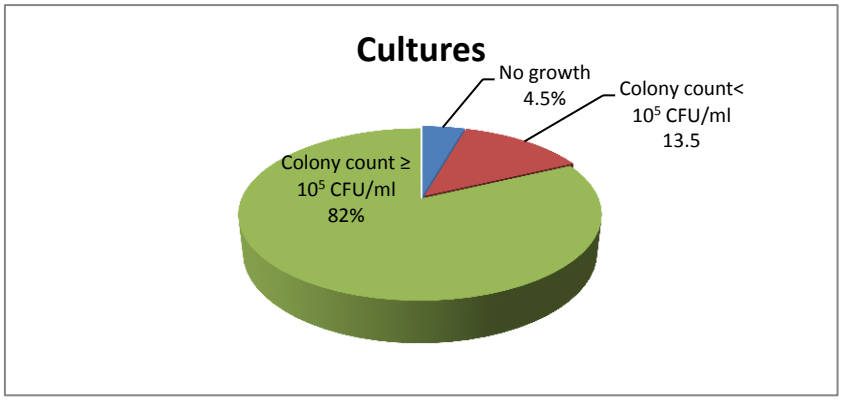

Fig 3: The results of cultures and cfu/ml of each culture

Table 5: MALDI Culture Score in different CFU cultures

	N	Mean	Std. Deviation	Minimum	Maximum
Less than 10^5 CFU/ml.					
Equal to or More than 10^5 CFU/ml.	164	2.17323	.102377	1.890	2.400
Total	191	2.17084	.110365	1.880	2.450
t-test 0.559 p value = NS					

The table shows that 164 samples (82%) yielded colony counts equal to or greater than 10^5 CFU/ml, 27 (13.5%) lower than 10^5 CFU/ml.

Table 6: Identified Organisms by MALDI-TOF from culture in relation to Gram stain Results.

	gram stain		Total
MALDICulOrg	Gram -ve Bacilli N (%)	Gram +ve Cocci N (%)	
A. baumanii	4 (2.1)	0	4
C. freundi	2 (1.0)	0	2
E. coli	107 (55.4)	0	107
E. cloacae	2 (1.0)	0	2
E. faecalis	1 (0.5)	3 (42.9)	4
K. pneumoniae	41 (21.2)	0	41
M. morgagni	1 (0.5)	0	1
P mirabilis	7 (3.6)	0	7
P. aeroginosa	18 (9.3)	0	18
S. aureus	0	4 (57.2)	4
Serratia	1 (0.5)	0	1
No growth	9 (4.7)	0	9
Total	193	7	200

Table 6 shows that MALDI-TOF after culture could identify 174 sample out of 193 of gram negative samples and the miss identified was yielded no growth.

Table 7: Frequency and percent of identification of organisms from culture.

	Frequency	Percent
Genus Level		
Klebsiella	1	.5
Pseudomonas	1	.5
Serraitia	1	.5
Staphyloccus	1	.5
Species level		
E.coli	107	53.5
K. pneumonia	40	20.0
P. aeruginosa	17	8.5
P. mirabilis	7	3.5
A. baumannii	4	2.0
E. faecalis	4	2.0
S. auerus	3	1.5
C. Freundi	2	1.0
E. cloacae	2	1.0
M. morgagani	1	.5
Total	191	100.0

The table shows that among correctly identified samples 191 (from culture) correctly identified 187 (98%) showed a species level identification score ≥1.99, 4 (2%) showed a genus level identification score >1.7 and <1.99.

Table 8: Identification of organisms by colours on culture media.

	Frequency	Percent
Identified	179	89.5
No growth	9	4.5
Not identified	12	6.0
Total	200	100.0

The table shows that we identified 179 (89.5%) samples out of 181 samples direct from cultures without need for further identification by any method.

Table 9: Cross tabulation test between direct and from culture methods.

		Culture		Total
		Genus level(culture)	Species level (culture)	
Diret MALDI-TOF	Not Identified (direct)	3	8	11
	Genus level (direct)	0	83	83
	Species level (direct)	0	91	91
	Insufficient (direct)	1	5	6
	Total	4	187	191
		Value	Asymp. Sig. (2-sided)	
Pearson Chi-Square		43.947[a]	.000	

The table shows that between 11 urine samples not identified by MALDI-TOF direct, after cultivation MALDI-TOF identified 3samples to genus level and 8 to species level. Of 6 samples with insufficient pellet in direct method, after cultivation MALDI-TOF identify 1 to genus level and 5 to species level.

The results of MALDI-TOF in relation to culture results were highly significant.

MALDI-TOF identified 174 samples direct from urine samples out of 191 samples identified from colonies after culture. MALDI-TOF directly identified 96 *E.coli* out of 107 after culture and 39 identified directly as Pseudomonas spp. out of 41 after culture.

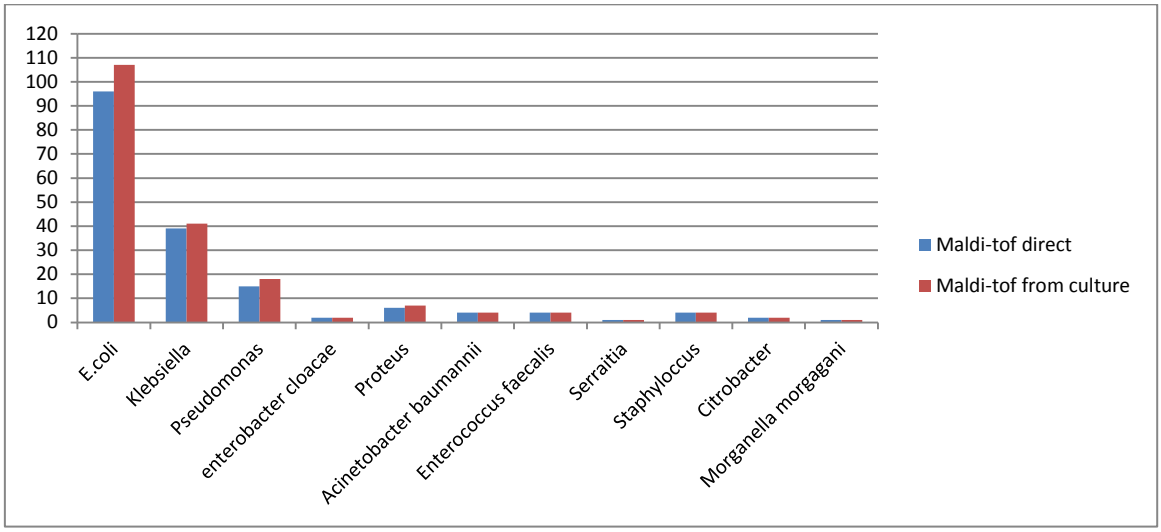

Fig 4: Comparison between MALDI-TOF direct and from culture.

MALDI-TOF after culture in comparison to both PCR and BD Phoneix:-

Table 10: shows identification of spp. By BD phoenix and PCR.

MALDI Culture Isolated Organisms	PCR and Bd Phoneix results							Total
	A. baumanii	E. coli	E. faecalis	P mirabilis	S. aureus	Pseudomonas	Klebssiella	
A. baumanii	1 (100.0)	0	0	0	0	0	0	1
E. coli	0	34 (100.0)	0	0	0	0	0	34
E. faecalis	0	0	3 (100.0)	0	0	0	0	3
K. pneumonia	0	0	0	0	0	0	25 (100.0)	25
P mirabilis	0	0	0	2 (100.)	0	0	0	2
P. aeroginosa	0	0	0	0	0	4 (80.0)	0	4
S. aureus	0	0	0	0	3 (100.0)	0	0	3
Pseudomonas	0	0	0	0	0	1 (20.0)	0	1
Total	1	34	3	2	3	5	25	73

The table shows that all 73 colonies from 73 samples give the same identification in MALDI-TOF after culture,Bd Phoneix and PCR.

Table 11: Sensitivity and specificity of MALDI-TOF direct method in comparison to MALDI-TOF after culture method.

		Culture		
		Identified	**Not Identified**	**Total**
Direct	**Identified**	174 (TP)	0 (FP)	**174**
	Not Identified	17 (FN)	9 (TN)	**26**
	Total	**191**	**9**	**200**
Sensitivity	91.10 %		Specificity	1OO.OO %
PPV	1OO.OO %		NPV	34.62 %

TP: True Positive, FP: False Positive, TN: True Negative, FN: False Negative.

The table shows that the sensitivity and specificity of MALDI-TOF identification by direct method was 91.0 and 100.0 compared to MALDI-TOF after culture. We can depend on direct MALDI-TOF in urine samples when we need to guide the physician to the empiric treatment until the full sensitivity test after 48h was obtained.

DISCUSSION

Urinary tract infections (UTIs) can present in a variety of clinical manifestations, ranging from bacteriuria with few symptoms to sepsis, severe sepsis, or septic shock[7].

In 20–30% of all septic patients the infection focus is localized in the urogenital tract. An adequate initial antibiotic therapy is essential since it ensures an improved outcome. Moreover, inappropriate antimicrobial therapy in severe UTI is linked to a higher mortality rate. For election of the empiric treatment in these cases, identification directly from urine samples of bacteria causing the infection can be useful to limit usage of last resort antimicrobials [8].

The standard quantitative urine culture takes 2–3 days, before results are available, clinicians often initiate treatment with antibiotics. These antibiotics are in many cases unnecessary, inefficient or cover a wider spectrum of microorganisms than necessary [9].

Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) technology is a technique which allows microbial identification in clinical microbiology laboratories based on microbial protein profiles. A promising application of the MALDI-TOF technique consists of carrying out identification from direct urine samples, without the need to cultivate them. In this way, a resultant microbiological species level can be obtained in less than one hour [10].

We found that Urine samples with counts of $\geq 10^5$ CFU/ml provided high identification scores(close to 2.000) and therefore high correct identification and this was similar to Gabriel et al.[10],The bacterial count seems to be a critical issue in obtaining good results with MALDI-TOF MS directly from clinical samples. The results obtained with clinical samples, when the bacterial count was $<10^5$ CFU/ml, were poorer than those obtained with high bacterial counts, While urine samples with bacterial counts of $\geq 10^5$ CFU/ml are almost always clinically relevant, urine samples with lower bacterial counts may be relevant or not. Thus, they should probably remain to be studied by conventional methods that allow measuring bacterial count, thus and manage the patient by taking this finding into account [10].

Another useful algorithm for the rapid diagnosis of UTI was presented by Burillo et al.[13], who combined Gram stain with mass spectrometry [2]. In accordance with Rodríguez-Sánchez et al.[11] and Ferreira et al. [12], the identification of Gram-negative bacteria has provided better results than Gram-positive and yeast. In our study we didn't get any yeast from urine direct cultures.We found an association between bacteriuria values provided by direct examination and microorganisms by MALDI-TOF MS. Data of bacteriuria provided by direct examination and gram staining guides the direct identification the same day of the sample collection, while conventional culture needs minimum 24-48 hours, an observation similar to Íñigo et al. [13].

In this study the correctly identified microorganism is 87%. The unreliable identification of the remaining 13% (10% unidentified and 3% insufficient pellet urines) could be related to low bacteriuria detected in these samples.

In general, microbial identification with MALDI-TOF from direct urine sample requires standardization of the procedure. Veron et al.[14,] compared different methods, concluding that a previous short culture and dual-filtration methods provided the best results[14]. We used the method as done by Ferreira et al. [12]. We didn't compare other methods.

In our study, we were able to predict the presence or absence of bacteriuria and the causative bacteria in patients with a suspicion of UTI rather accurately and within a working laboratory shift by performing a Gram stain followed by MALDI-TOF MS immediately on urine samples. This was in agreement with Burillo et al.[2], who stated that physicians would receive therapeutically helpful information for 96.1% of samples in less than one hour.

The BD Phoenix correctly identified 73 (100%) isolates to the species level, similar to the results we got from MALDI-TOF MS. This is in accordance with Saffert et al.[3], who got 93% of the common isolates to the species level from both systems. However, they found that overall the Bruker Biotyper exhibited better performance for identifying isolates to the genus and species level (93% and 82%). This difference can be attributed to the improved performance of the Bruker Biotyper in identifying isolates infrequently isolated in the clinical laboratory. Our lack of similar findings could be explained by only having common strains and only from urine samples, but Saffert et al.[3], included different strains from many sources .

The Bruker Biotyper MALDI-TOF MS outperformed the BD Phoenix Automated Microbiology System for Gram negative bacteria infrequently cultured in the clinical laboratory. However, for common isolates, the systems performed equivalently.

The multiplex PCR correctly identified 73 (100%) isolates to the species level as the results obtained by Maldi-Tof . This is similar to Cherkaoui et al. [15], who compared two Matrix-Assisted Laser Desorption

Ionization-Time of Flight Mass Spectrometry Methods (Bruker and Shimadzu) for Routine Identification of bacteria to the species level and found both systems to be >99% accurate compared with PCR[15].

With the improved turnaround time and cost effectiveness of the Bruker Biotyper system, MALDI-TOF MS technology provides an advance in bacterial identification in the clinical microbiology laboratory.

In our results sensitivity of direct method was 91.10% and specificity 100% this was similar to Huang et al.[16], sensitivity of was 93.4% and specificity 96.3%.

In conclusion, the combination of urine screening methods, such as microscopic urine analysis, dipstick examination and MALDI-TOF MS provided a reliable bacterial identification from infected urine, whose shortened analysis time enables an earlier and more accurate selection of antibiotics for patient treatment.

Our study showed some limitations. The small number of patients (200) over a long period of time (12 months) in only one laboratory and it is outpatient laboratory is an important limitation that could compromise different spp. related to hospital acquired infection. However, Central lab of armed force is a representative large laboratory in EGYPT.

Second, we relied on physicians lab request, i.e, we did not include routine cultures at specified times during the study design.

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This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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