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Direct identification of bacteria in urine samples by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and relevance of defensins as interfering factors

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Standard methods for the identification of uropathogens that are based on the determination of metabolic activity require cultivation on agar plates, which often takes more than 1 day. If microbial growth on agar plates is slow, or if metabolic activity is impaired by adverse interactions resulting from the patient's condition or from medical treatment, the application of standard methods may lead to delayed or erroneous identification of bacteria. In recent studies, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has proven to be able to rapidly identify bacteria obtained from cultures. We tested the applicability of this analytical technique for the rapid identification of bacteria collected directly from urine samples and compared the results with those of conventional identification methods, such as the Vitek system, the MicroScan WalkAway system and the API system, and in some cases with the gas chromatographic determination of the bacterial long-chain fatty acid pattern. We analysed a total of 107 urine samples with bacterial counts ranging from 10^2 to $\geqslant 10^5$ c.f.u. ml^{-1}. Mass spectrometric identification of bacteria was accomplished for 62 of these samples. In the mass spectra obtained from 40 of the 45 urine samples for which no identification result was achieved, a triplet of very intense peaks corresponding to the human α-defensins 1, 2 and 3 occurred at *m/z* values of around 3440 Da. This signal suppressed the intensity of the bacterial protein peaks and thus impaired database matching. Our results show that MALDI-TOF MS allows the reliable direct identification of bacteria in urine samples at concentrations as low as 10^3 c.f.u. ml^{-1}. In a subset of samples, human defensins may occur and impair the mass spectrometric identification of bacteria.

INTRODUCTION

The classic definition of a urinary tract infection (UTI) is a bacteriuria, at a bacterial count of $\geqslant 10^5$ c.f.u. ml^{-1}, in combination with clinical symptoms. Even bacterial counts as low as 10^2 or 10^3 c.f.u. ml^{-1} may be clinically relevant if they indicate insufficient drug treatment or a developing UTI for instance (Grabe *et al.*, 2008a, b). To identify the uropathogens by standard methods, amplification by growth on an agar plate is necessary, which often requires a minimum of 24 h. Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been introduced as a new analytical approach to rapidly identify bacteria obtained from cultures. This method has proved its applicability in various studies, and it is already being used in some laboratories as a routine procedure. The direct identification of uropathogens in urine by MALDI-TOF MS has also been reported whereby bacterial counts of 10^5 c.f.u. ml^{-1} have been considered as the lower limit for identification (Maier *et al.*, 2008; Ferreira *et al.*, 2010). The goal of the present study was to develop a

Abbreviations: CoU, Clinic of Urology; DHB, dihydroxybenzoic acid; IMMi, Institute of Medical Microbiology; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SARAMIS, Spectral Archive and Microbial Identification System; UTI, urinary tract infection.

protocol for efficiently separating bacteria from the urinary matrix and directly identifying them by MALDI-TOF MS even where the bacterial count is lower than 10^5 c.f.u. ml^{-1}. Because confidence levels for MALDI-TOF MS identification of bacteria become lower with decreasing numbers of bacteria on the MALDI spot, an appropriate protocol must include the isolation of sufficient bacterial cells and the depletion of contaminants. According to the supplier of the Spectral Archive and Microbial Identification System (SARAMIS), the identification system we used, a mass spectrum quality necessary for the highest possible confidence level can be achieved if the MALDI spot contains 10^6 bacteria or, for some species, 10^5 bacteria (AnagnosTec, unpublished data). Here we present identification results obtained by an isolation protocol that has been optimized with regard to maximum accessible confidence levels and the lowest expenditure of time required for analysis. We have compared the results with those obtained by standard identification procedures and, in specific cases, by the gas chromatographic determination of bacterial long-chain fatty acids.

METHODS

Pre-analytic preparation of urine samples. We analysed 107 urine samples collected from inpatients of the University Hospital Essen for bacterial content by using MALDI-TOF MS in our institute. Prior to that, all samples had been analysed by standard procedures in the submitting laboratories, the Institute for Medical Microbiology (IMMi) or the Clinic of Urology (CoU), including cultivation on blood agar and MacConkey agar plates inoculated with a 10 µl loop, as well as Gram staining and testing for oxidase and catalase activity before identification by the Vitek system (bioMérieux) or by the MicroScan WalkAway system (Siemens Healthcare Diagnostics). If the identification results conflicted with bacterial characteristics such as Gram staining behaviour, (im)mobility, antibiotic resistance and (an)aerobic growth or if identification scores were poor (<80 %), the bacteria were identified by the API system (bioMérieux) or by the gas chromatographic determination of the bacterial long-chain fatty acid pattern (Hewlett Packard GC System 5890 Series II GC; Microbial Identification System, MIDI) (Müller *et al.*, 1990). Only results that were concordant with bacterial characteristics and had a minimum score of 80 % were copied into the laboratory information management system). We were blinded to the identification result obtained by either the IMMi or the CoU. First, we performed an orienting cell count and roughly classified all bacteria-containing samples with regard to sediment other than micro-organisms. Most of the urine samples received from the CoU were characterized by a prominent macrohaematuria and by more cell detritus than that contained in the samples obtained from the IMMi. The urine samples were poured into 15 ml conical tubes and centrifuged for 5 min at 1000 ***g*** and 20 °C to separate the nonbacterial contaminants from the micro-organisms. The contaminants accumulated in a pellet, whereas the bacterial count in the supernatant decreased only marginally. The supernatant was decanted into a filtration device [Millipore Microfil S Filtration Device with a polycarbonate track etch filter membrane (Polycarbonate Track Etch Membrane Filters, type 23007, diameter 47 mm, pore size 0.2 µm; Sartorius Stedim Biotech)]. After the passage, 3 ml sterile 0.9 % sodium chloride solution was added while filtration continued to dilute potentially interfering proteins. To suspend bacteria from the filter the membrane was put into a 1.5 ml conical centrifuge tube and vortexed for 1 min in 1 ml sterile deionized water. The supernatant was then centrifuged for 10 min at 18 000 ***g*** and 20 °C. The supernatant was withdrawn, and the pellet, consisting primarily of bacteria, was allowed to dry in ambient air for several minutes before a pipette tip was used to transfer it to the MALDI-TOF MS target plate. Subsequently, 0.3 µl 2.5-dihydroxybenzoic acid matrix solution (DHB Matrix Solution; AnagnosTec) was added. If the pellet was too small to be picked up, it was dissolved in 10 µl DHB, and 0.3 µl of this solution was then pipetted onto one position of the target plate at a time. As a minimum, duplicates on the target were prepared from each sample. After crystallization, the target was transferred into the MALDI-TOF MS for analysis.

Mass spectrometric analysis. All experiments were performed on a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems) equipped with a nitrogen laser (wavelength, 337 nm; pulse length, 3 ns; maximum firing rate, 20 Hz) in the linear mode with an effective path length of 1.35 m (mass accuracy $\pm$0.05 % for angiotensin and myoglobin for external calibration). The spectrometer was controlled by the Voyager 5 software. According to the recommendations of the manufacturer of the SARAMIS software (AnagnosTec), the spectra were calibrated externally by using a protein standard prepared from *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919 (DSM 1576, ATCC 8739, NCIB 8545). As a minimum, 200 laser shots were collected for each spot. The processing of the spectral data by the Data Explorer 4.6 software (Applied Biosystems) included baseline subtraction, noise reduction and peak detection implemented in a macro programmed by AnagnosTec. The SARAMIS identification system compares the generated peak lists with its database containing more than 2600 SuperSpectra and 35 000 single fingerprint spectra of various isolates. These spectra represent more than 2000 species and 500 genera; the pattern recognition algorithm used refers to peak position, peak intensity distributions and peak frequencies. SuperSpectra are generated on the basis of measurements of 15–20 different isolates representing the same species that have been well identified by reference methods. To each of the more than 30 *m*/*z*-values of the reference spectrum (fingerprint spectrum or SuperSpectrum), a weight, ranging from 0 to 40 points, is attributed that represents the species specificity of that mass. The confidence level of a spectrum is simply the sum of all points of all matching masses. If the sum is 999 or more, the result is considered confirmed at a 99.9 % confidence level. In the default settings, only results with a minimum confidence level of 75 % are displayed. According to the software provider, identifying bacteria at the species level requires a confidence level of at least 80 %, whereas identification at the family level is possible even at a confidence level of 75 %. The provider has optimized the process of database matching for bacteria picked up from the agar plate in that the peak exclusion list contains masses of constituents of commonly used agars that are irrelevant for species identification. If bacteria grow in urine, they may contain proteins that are different from those present in agar; thus, their peaks may not be excluded in the default settings of the SARAMIS software.

RESULTS

Of the 107 urine samples analysed by MALDI-TOF MS, the 55 samples sent to the IMMi were collected from inpatients of several clinical departments; 52 samples originated from inpatients of the CoU. Because we were blinded to the results, we did not know the bacterial counts that had been estimated by the use of a cell count chamber in the CoU laboratory or by the enumeration of c.f.u. after inoculation of culture plates in the IMMi, nor did we know the identification result obtained by standard methods. After

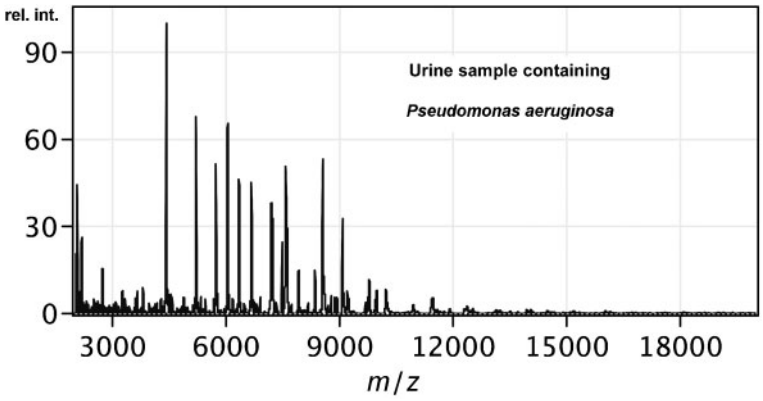

Fig. 1. Fingerprint mass spectrum of *Pseudomonas aeruginosa* from a urine sample containing no defensins.

blinding was removed, the results showed that a number of the urine samples sent to the IMMi contained relatively few bacteria (<500 c.f.u. ml^{-1}). Large numbers of erythrocytes were rarely found in samples sent to the IMMi but were quite common in urine samples collected from inpatients in the CoU. Twenty-six (50 %) of the isolates obtained from the CoU and 36 (69 %) of the isolates sent to the IMMi were identified unequivocally by MALDI-TOF MS, including six samples with completely different results to those obtained by routine methods. For three isolates (6 %), the MS-based procedure identified only the family. MALDI-TOF MS analysis detected three mixed bacterial infections in urine samples with a total bacterial concentration of $\geqslant 10^5$ c.f.u. ml^{-1}, whereas routine analysis detected 15 mixed infections and 21 contaminations in samples with this bacterial concentration. In 10 of the 15 CoU samples that contained mixed bacterial infections, only one species was found at a concentration of 10^5 c.f.u. ml^{-1}. Therefore, it was impossible to distinguish these samples from contaminated samples.

A common feature of the mass spectra recorded from 22 of the 26 CoU samples in which the urinary pathogen could not be identified by MS analysis was a triplet of very intense peaks with *m*/*z* values of 3371.0, 3442.5 and 3486.5 Da. This peak triplet completely depressed the signals referring to the fingerprint spectrum (see Figs 1 and 2). Of the remaining four CoU urine samples with unidentified bacterial infection, one yielded a poor mass spectrum without that triplet and three yielded negative results from both MS analysis and analysis in the CoU laboratory.

Bacterial identification is routinely performed in the CoU laboratory only if the urinary cell count is at least 10^5 c.f.u. ml^{-1}, as estimated by the use of a cell count chamber. In only one of the analysed CoU samples was the bacterial concentration as high as 10^4 c.f.u. ml^{-1}. Because of incomplete documentation, the c.f.u. number remained unknown for eight samples. Urine samples sent to the IMMi from various clinical departments are not preselected by cell count; thus, their bacterial concentrations ranged from 10^2 to 10^5 c.f.u. ml^{-1} (Table 1).

The very intense triplet mentioned above was present in only eight of the 52 spectra recorded from samples sent to the IMMi. Again, the bacteria in these samples could not be identified by MALDI-TOF MS. The overall fraction of successful identification was higher. In three of the 15 urine samples containing 10^3 c.f.u. ml^{-1}, no spot could be obtained on the MALDI target because of poor harvesting or crystallization. The results are shown in Tables 1 and 2.

DISCUSSION

The results indicate that micro-organisms in urine can be directly identified by MALDI-TOF MS within 30 min and, at least in a subset of samples, at bacterial concentrations as

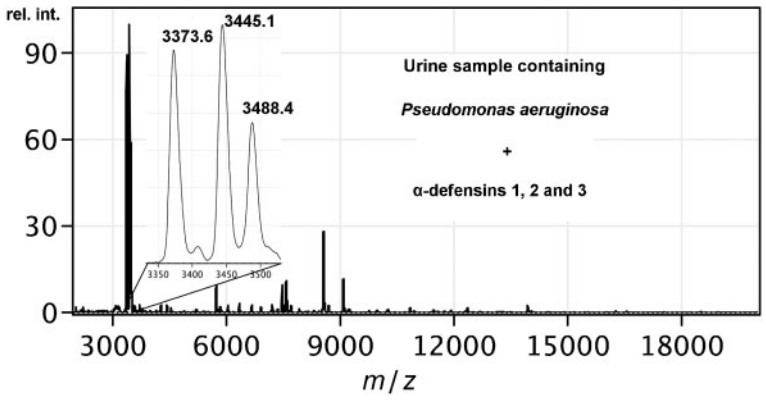

Fig. 2. Spectrum of *Pseudomonas aeruginosa* suppressed by high-intensity peaks of human α-defensins 1, 2 and 3 (*m*/*z*: 3373.6 Da, 3445.1 Da and 3488.4 Da).

Table 1. Identification results obtained by direct MALDI-TOF MS analysis of bacteria in urine samples from inpatients

Results are classified according to bacterial count and origin of the samples.

Bacterial concn (c.f.u. ml^{-1})	Origin of samples	Total no. of samples	Samples without defensins [*n* (%)]	Identified samples without defensins [*n* (%)]	SARAMIS confidence level			
					99.9 %	**99.8–80.0 %**	**79.9–75.0 %**	**<74.9–55.0 %**
$\geqslant 10^5$	CoU	31	18 (58)	18 (100)	4 (22 %)	4 (22 %)	0	10 (56 %)
	IMMi	19	17 (90)	16 (94)		4 (25 %)	4 (25 %)	8 (50 %)
$\geqslant 10^4$	CoU	2	1 (50)	1 (100)				1 (100 %)
	IMMi	12	10 (83)	10 (100)	1 (10 %)	2 (20 %)	3 (30 %)	4 (40 %)
$\geqslant 10^3$	CoU	/						
	IMMi	14	12 (86)	6 (50)		3 (50 %)	1 (17 %)	2 (33 %)
$\geqslant 10^2$	CoU	/						
	IMMi	5	3 (60)					
Unknown	CoU	19	11 (58)	9 (82 %)	2 (22 %)	2 (22 %)	1 (11 %)	4 (44 %)
	IMMi	3	3 (100)	3 (100 %)	1 (33 %)	2 (67 %)		

low as 10^3 c.f.u. ml^{-1}. The presence of erythrocytes and sediment components other than bacteria did not significantly impair the identification. In cases of mixed infections, more than one strain of bacteria could be identified by MALDI-TOF MS if their concentrations were at a similar order of magnitude. Highest confidence levels were achieved if the bacterial concentrations ranged from 10^4 to 10^5 c.f.u. ml^{-1}. Bacteria present in low concentrations, such as those that typically originate from contaminated indwelling catheters, did not impair the identification of the uropathogens responsible for the UTI. Unlike Ferreira *et al.* (2010), we adhered to the protocol described above and did not include additional extraction steps, even if the filtration method did not yield bacteria in a quantity sufficient for mass spectrometric identification.

We occasionally encountered problems resulting from the allocation of urine volumes of less than 3 ml, from the storage of samples for extended periods of time, or from inadequate storage of the samples at room temperature. However, the most important impairing factor proved to be the appearance of proteins resulting in a triplet of very intense peaks at *m*/*z* values of 3371.0, 3442.5 and 3486.5 Da, which impaired the quality of the fingerprint spectrum. These mass-to-charge ratios correspond to those of the human α-defensins 1, 2 and 3 (Zhang *et al.*, 2002). They suppress the peaks reflecting the bacterial proteins and therefore inhibit database matching.

Human α-defensins 1, 2 and 3 are composed of 29 or 30 amino acids with a relatively high fraction (24 %) of the basophilic arginine (R), glycine (G) and histidine (H), which have a high proton affinity. The ionization of proteins by matrix molecules in the matrix-protein plume after the laser shot is dominated by proton transfer and prefers those proteins with a high proton affinity to the disadvantage of proteins with only an average content of R, G and H (14 %) and therefore a lesser proton affinity. It may be this effect that suppresses the ionization and peak intensity of the bacterial proteins when defensins occur in the sample (Knochenmuss, 2006).

There are reports of increased concentrations of defensins in patients with pyelonephritis (Hiratsuka *et al.*, 2000) or cancer of the bladder (Holterman *et al.*, 2006; Vlahou *et al.*, 2001). We were informed that most of the samples from the CoU were collected from patients after surgery of the lower urinary tract, and we found that the percentage of haematuria was higher in these samples than in the samples sent to the IMMi. The fact that defensins were present particularly in a subset of samples originating from patients who had undergone urological surgery may explain why this important confounder in the identification of bacteria by MALDI-TOF MS has not been previously reported.

Our protocol for the isolation of bacteria does not allow detachment of the bacteria from defensins. The amphipathic properties of defensins enable them to interact with microbial membranes. Because of electrostatic attraction, the transmembrane bioelectric field draws the defensins

Table 2. Frequency distribution of species identified in urine samples by MALDI-TOF MS

Species	No. of isolates	Mean identification score (highest–lowest score)
Escherichia coli	30	76 % (99.9–57 %)
Family *Enterobacteriaceae*	9	77 % (99.9–55 %)
Klebsiella pneumoniae	4	85 % (97–76 %)
Pseudomonas aeruginosa	4	84 % (99.9–58 %)
Citrobacter freundii	3	67 % (72–62 %)
Proteus mirabilis	3	92 % (99.9–84 %)
Enterobacter cloacae	2	65 % (73–57 %)
Enterobacter aerogenes	1	76 %
Enterococcus faecalis	1	88 %
Enterococcus sp.	1	74 %
Klebsiella oxytoca	1	67 %
Klebsiella sp.	1	70 %
Morganella morganii	1	94 %
Proteus vulgaris	1	75 %
Serratia marcescens	1	73 %
Serratia sp.	1	60 %
Stenotrophomonas maltophilia	1	63 %

toward and into the bacterial membrane (Ganz, 2003). Therefore, defensins cannot be removed simply by washing the filter residue.

The recent publication of Ferreira *et al.* (2010) presents spectra of samples that yielded no reliable identification result. These spectra show typical defensin peaks; however, the authors did not reveal the identity of these peaks.

The occurrence of peaks representing proteins not originating from bacteria that do not suppress the fingerprint spectrum does not impair database matching. Such matching is accomplished by an algorithm in the SARAMIS software that calculates the confidence level by adding points of matching peaks (AnagnosTec SARAMIS Manual, version 1.3.3_2, 2009). The presence of peaks primarily in the low *m*/*z* range, which do not correspond to bacterial proteins, does not decrease the confidence level. Thus, there is no need to define new lower score limits for identification if the bacteria originate from a biological matrix other than the agar plate. In some studies of direct identification by MALDI-TOF-MS in which a direct identification of bacteria in blood or urine by MALDI has been reported, an increased proportion of bacterial identification results from a deliberate and unjustified decrease of score limits by the authors. Whereas SARAMIS uses a linear score called confidence level expressed as %, which equals one-tenth of the accumulated points of matching peaks with an upper cut-off of 999 points, the MALDI Biotyper (Bruker Daltonics) classifies the results using a score that is the logarithm of the points calculated by a different algorithm. According to instructions by Bruker, the identification of bacteria requires not fewer than 10^5 cells per spot (Maier *et al.*, 2008) and is 'highly probable' at the species level if the score is in the range of 2.3–3, corresponding to 200–1000 points, and only 'probable' in the range of 2.0–2.299, corresponding to 100–199 points. No identification of the species is achieved if the score is between 1.7 and 1.99, corresponding to 50–99 points.

In the study by Ferreira *et al.* (2010), the identification of bacteria by MALDI-TOF MS and the MALDI Biotyper was classified by the authors using new score limits below those defined by Bruker and a different designation, but no compelling evidence was given. The species identification was considered 'reliable' instead of 'probable' at a score of $\geqslant 2$ ($\geqslant 100$ points) and the genus was considered identified if the logarithmic score was between 1.7 and 1.9 (50–99 points). This may explain why the authors report that they regard 87 % (205 of 235) of all samples containing bacteria at a concentration of $>10^5$ c.f.u. ml^{-1} (with one exception) as identified at the species level. SARAMIS was optimized to prevent false-positive results in the automatic mode; therefore, species identification is considered 'unequivocal' only within 1000–1400 points, expressed as a confidence level of 99.9 %. A 'reliable' species identification requires 800–998 points, expressed as a confidence level of 80–99.8 %. At a confidence level of 75–79.9 %, the species identification is considered 'probable'. No confidence level is provided if the confidence level is below 75 %. We did not change the identification limits, but we did set SARAMIS to display results even if the confidence level was as low as 55 %. If we exclude samples containing defensins, SARAMIS identified 94 % of the samples if the bacterial count was $\geqslant 10^5$ c.f.u. ml^{-1} and all samples if the bacterial count was $\geqslant 10^4$ c.f.u. ml^{-1}. With a bacterial count of 10^3 c.f.u. ml^{-1}, a result was achieved in only 55 % of the samples. Although the confidence level was below 75 % in 17 samples with a bacterial count of $\geqslant 10^5$ c.f.u. ml^{-1}, the results of only four samples differed from those obtained by standard methods. In five samples with a

bacterial count around 10^4 c.f.u. ml^{-1}, the confidence level was below 75 %, but the identification result was still consistent with that obtained by standard methods. The same holds true for samples with a bacterial count of $\geqslant 10^3$ c.f.u. ml^{-1}, although a confidence level of 99.9 % was not reached in any of these samples. It appears that the achievable confidence level does not depend on the presence of contaminants such as detritus, on bacterial species in low concentrations originating from the catheter, or on the presence of low-abundance proteins in the sample with average proton affinity. It probably depends to a greater degree on the number of bacterial cells on the MALDI spot and, therefore, on the bacterial count in the sample. A confidence level of 99.9 % generally requires the deposition of 10^5–10^6 cells on the MALDI spot. Such a quantity is not available if the sample volume is small and the bacterial count is low. However, even though the confidence level is low in such cases, SARAMIS was able to correctly identify the isolates because of its sophisticated algorithm based on SuperSpectra, and it did not offer numerous competitive pseudo-hits.

Many urine samples sent to the IMMi were taken from patients who had undergone bone marrow transplant and had a long history of antibiotic treatment. Therefore, the identification of the bacteria by standard procedures often required several days because of reduced microbial growth or altered enzyme activity. In many of these cases, only gas chromatographic identification of the long-chain fatty acid pattern yielded an unambiguous result. In contrast, MALDI-TOF MS identified the bacteria in these samples in under 30 min.

In contrast to a statement in a recent review (Bizzini & Greub, 2010), we found that it is not necessary to cultivate the bacteria before they are directly identified by MALDI-TOF MS if the bacterial concentration in the urine is in the range of 10^3–10^5 c.f.u. ml^{-1} (Borovskaya, 2009; Kostrzewa *et al.*, 2010). In addition, our results indicate that direct MALDI-TOF MS identification of bacteria in urine samples can be performed even in the presence of more than two uropathogens. The method makes it possible to begin early species-specific antimicrobial treatment, the effectiveness of which can be monitored within narrow time intervals. This early treatment reduces the likelihood of complications and the costs of patient care.

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