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REVIEW ARTICLE

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry as Internship Teaching Content in Laboratory Medicine

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SUMMARY

Background: **Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) is one of the preferred detection techniques for identification of clinical microorganisms and it has the characteristics of rapid identification, simple operation, low cost, and updatable databases. For laboratory medicine undergraduates, clinical internship is an important stage for the connection of basic theoretical knowledge and clinical practice. Internship teaching choosing MALDI-TOF MS as the content will greatly increase the popularity and applicability of the new technology in the clinical microbiology laboratory.**
Methods: **With the help of electronic databases on the network, we conducted a systematic review. According to the purpose of research, we singled out forty papers. Latest studies on history, basic principles, clinical features, and applications of MALDI-TOF MS and the internship teaching contents introducing new technologies are summarized and focused on. In internship teaching, firstly we explain the historical development, basic principle and widespread applications of MALDI-TOF MS in the identification of clinical pathogenic microorganisms and the detection of antibiotic resistance. Subsequently, we instruct the students to perform the experimental operations, analyze the common problems, and find solutions. Finally, we highlight quality control and laboratory biosafety.**
Results: **Most of the reviews published previously report the clinical features and applications of MALDI-TOF MS and the internship teaching contents choosing other new technologies. It is the first study selecting MALDI-TOF MS technology as an internship teaching content creatively. Primary outcome is that the students understand the theoretical knowledge in detail, master the operation skills of MALDI-TOF MS quickly, and obtain excellent internship performances in the clinical internship through the internship teaching. Secondary outcome is that it is a help to cultivate medical students' train of thought for scientific research and to understand the application of the new technology in clinical testing and scientific research.**
Conclusions: **Laboratory medicine undergraduates should cherish the opportunity to learn the new technology during the internship period and should master basic principle and operation. As internship teachers, it is necessary to introduce the new technology to students during the internship and encourage undergraduates to cultivate creative and innovative thinking of scientific research.**
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KEYWORDS

MALDI-TOF MS, clinical internship, internship teaching content, clinical microbiology, laboratory medicine

INTRODUCTION

Microorganisms generally mean living organisms (i.e., bacteria, fungi, protozoa, and viruses) ranging in size from a few millimeters to nanometers. They are always ubiquitous because that they have adapted to harsh and extreme environments and diverse metabolic and survived. It is considered that a drop of water might consist of millions of bacteria or even more [1]. Accurate and rapid detection of pathogens is required in the department of medical laboratory, especially for clinical microbiological testing. Though many standard physiological, morphological, and biochemical techniques for microbiological detection demonstrate strong differentiation power based on bacterial strains growing on culture medium, the traditional methods always have several shortcomings. They are labor intensive, time-consuming, complex, and costly. A variety of molecular methods based on nucleic acids including real-time polymerase chain reaction (RT-PCR), 16s rRNA, and next generation sequencing (NGS) have also been developed and present powerful detection abilities. However, these sophisticated techniques require high expertise [2]. MALDI-TOF MS is an advanced technology which can create a unique mass spectrum of microorganisms by the detection of mass to charge ratio (abbreviated as M/Z) of ribosomal proteins in a minimum amount of time. The identification depends on a set of ion peaks of a distinctive mass spectrum which constitutes a fingerprint. The detection is accomplished by matching the spectrum with the spectra of reference strains collected in the databases [3]. MALDI-TOF MS becomes a preferred technique in detecting pathogenic microorganisms, which is of great value in the clinical microbiology laboratory [4,5]. Laboratory medicine is a science that provides information for the prevention, diagnosis, and treatment of human diseases and the evaluation of human health status by conducting microbiological, immunological, biochemical, and hematological tests on human specimens. It is a subject closely combining theory with practice and is a highly practical applied specialty [6]. For laboratory medicine undergraduates, clinical internship is an important stage for the connection of basic theoretical knowledge and clinical practice and a key stage for the accumulation of clinical practice and the improvement of their comprehensive ability. Clinical internship will have a direct impact on their future work ability and could affect their employment after graduation [7]. Internship teaching is an important teaching stage and introducing MALDI-TOF MS into the internship teaching contents will improve the understanding, operation ability, and acceptance of the new technology to interns. It will greatly increase the popularity and applicability of MALDI-TOF MS. Familiarity with MALDI-TOF MS in the clinical microbiology laboratory is of great significance for interns to opt for a career in clinical work or scientific research [8].

MATERIALS AND METHODS

According to the purpose of our study, forty papers were sorted out from literature databases on the network. In the review, latest studies on history, principles, clinical features, and applications and the internship teaching contents introducing new technologies are summarized in detail. During internship teaching, we first explain the historical development and basic principle of MALDI-TOF MS, its widespread application in the identification of clinical pathogenic microorganisms, and the helpful detection of antibiotic resistance. Subsequently, we instruct the students to perform the experimental operations, analyze the common problems, and find solutions. Finally, we highlight the quality control and laboratory biosafety.

History and development of MALDI-TOF MS

In 1975, Anhalt first proposed the application of MS technique to detect bacteria. Due to technical limitations, the ionization method used could only fragment proteins and effective analysis failed to complete [9]. In the 1980s, Tanaka Koichi shared the 2002 Nobel Prize in Chemistry with John Fenn. Tanaka Koichi developed electrospray ionization technology and John Fenn invented the soft desorption ionization method for MS. They developed the application of laser desorption/ionization technology detecting biological macromolecules [10]. Meanwhile, Franz Hillen Kamp and Michael Karas were exploring the use of nicotinic acid as a matrix [11]. The MS fingerprints of whole bacteria ribosomal proteins were acquired for the first time in 1996 employing the MALDI-TOF MS technique [12]. Soon afterwards the detection of pathogens received more and more attention [13]. In the last 13 - 16 years, MALDI-TOF MS upgraded to a first-line detection method in labs and has been routinely utilized in Europe, North America, and Asia. It meets the needs of reliability and rapidness in the identification of clinical microorganisms. Simple operation, rapid identification, low cost, and updatable databases are the key features. Detecting time is also reduced from several days to minutes or even seconds [14].

Basic principle of MALDI-TOF MS

The MALDI-TOF MS equipment mainly consists of matrix assisted laser desorption/ionization (MALDI) ion source and time of flight (TOF) analyzer. MALDI-TOF completes the co-crystallization of sample and matrix formed by laser irradiation and then the ionized sample accelerates through the vacuum flight pipeline under the action of an electric field which is detected according to the different flight time. Molecular weight of sample

can be measured by M/Z which is proportional to the flight time.
MALDI-TOF MS is an excellent tool for bacterial identification and MS fingerprints are used to identify and group bacteria. Different microorganisms have specific protein fingerprints due to different molecular weight. Fingerprints are automatically matched with those of the known reference bacteria strains in the databases, and they are identified according to the comparison scores. Fingerprints mainly contain M/Z peaks of ribosomal proteins which have relatively high content in bacteria cells. Ancient molecules always exist in ribosomal protein and more than one third of ribosomal protein families express conservatively in Archaea, Bacteria and Eucarya. Bacteria can be classified on the basis of ribosomal proteins and the classification can be subdivided down to the genus, species, and even subspecies levels (Figure 1) [15]. In a study by Ceballos-Garzon et al., a total of 470 blood cultures were detected by MALDI-TOF MS, and good identification results (420/470, 89%) were obtained, including 283/470 (60%) identifications at species and genus level and 137/470 (29%) identifications at genus level [16,17].

Application of MALDI-TOF MS in the identification of clinical pathogenic microorganisms and antibiotic resistance

Identification of aerobic and anaerobic bacteria

The identification of common aerobes, such as *Staphylococcus*, *Enterococcus*, *Enterobacteriaceae*, *Campylobacter* and *Vibrio* using MALDI-TOF MS is accurate and reliable [18,19]. For some rare species, clinical laboratory experts spend about four days to make confirming diagnoses by isolation and detection with routine methods, including inoculation on universal and selective mediums, gram stain tests, biochemical tests, and antimicrobial susceptibility tests. The time of identification could be reduced to two days with MALDI-TOF MS. Watthanaworawit et al. created a routine direct spotting method to identify *Burkholderia pseudomallei* fast and cheap through spectra and superspectra. Identification of anaerobes is also always recognized as a difficulty in clinical microbiology, and traditional phenotypic identification methods are still deficient in the accuracy [20]. The new technique improves accurate detections of anaerobes, such as *Clostridium*, *Bacteroidetes*, *Prevotella* and so on, which is of great value to guide rational drug use for clinicians. Bachli et al. reported that 155 anaerobic bacteria strains from human clinical specimens were isolated and identified using traditional methods and MALDI-TOF MS simultaneously. Comparing the frequencies of anaerobic species from 2008 to 2012, which was prior to the implementation of MALDI-TOF MS, to those from 2013 to 2020 when MALDI-TOF MS came into use, they found that the species of anaerobic bacteria increased rapidly. Twenty species were detected in 2012, and there were 31 species in 2013. When MALDI-TOF MS became the preferred method of identification in 2020, the number of species reached 41 [21]. Clinical and scientific research databases still need to be optimized and improved by constructing protein fingerprints of new species and increasing the number of effective peaks for each species [22].

Identification of *fungi* and *mycobacteria*

With a growing number of patients with immune deficiency, the incidence of invasive fungal infection has gradually increased, and morbidity and mortality have shown an upward trend in the departments of Hematology, Respiratory, Infection, and ICU. The difficulty in early diagnosis, rapid progress of the disease and high cost of treatment have great impacts on patients, their families, and even the whole society. Traditional phenotypic identification is time-consuming and requires a high level of professional knowledge. MALDI-TOF MS can accurately identify common and uncommon clinical yeasts, including *Candida Tropicalis*, *Candida Parasmooth*, *Candida Portuguese* and so on. Bonifaz et al. evaluated 304 yeast isolates. They were mainly from superficial and deep mycoses. There were 277 in 304 (91.2%) strains using MALDI-TOF MS. The specificity and sensitivity of diagnosis of pathogenic yeasts in clinical samples were 99.0% and 94.6% respectively [23]. The new technology almost replaces the traditional pathogenic yeast identification methods with the advantages of being simple, fast, and reliable [24]. For some uncommon fungi that are difficult to identify, new methods are constantly being invented. Kocurek et al. applied liquid extraction surface analysis mass spectrometry (LESA-MS) to release intact fungi proteins rapidly with electroporation. Electroporation had a home-built high-voltage device which was designed to lyse cells grown in colonies on agar media [25]. However, MALDI-TOF MS still has some limitations in the identification of filamentous fungi which is unable to be identified or identified incorrectly due to fewer protein fingerprints and higher error rate. Researchers often improve the identification rate through self-built databases and special protein extraction methods. *Mycobacterium* always has its slow growth, long culture period, and harsh growth conditions. Routine identification methods mainly rely on biochemical reactions and DNA probes. Gene sequencing needs strict requirements for operators, equipment, and environment. They are cumbersome and expensive [26,27]. Fernandez-Esgueva et al. evaluated the identification of *Mycobacterium tuberculosis* and *non-tuberculous Mycobacteria* (NTM) by MALDI-TOF MS from January to December in 2017. Among 155 isolates in all, 152 isolates (98.06%) had convincing results [28].

Direct identification of clinical microbiological specimens

Clinical microbiological specimens can be directly identified including positive blood cultures, midstream urine, cerebrospinal fluid, pleural and abdominal fluid, and articular fluid. The direct identification of mid-

stream urine and positive blood cultures are reported most often. In positive blood cultures, the turnaround time for blood pathogens identification is roughly 24 - 48 hours. When the earlier treatments cannot be given, an empirical antibiotic regimen is prepared for the patients with sepsis, what is much worse, some patients are not treated at all. Statistics indicate that each hour of delay in appropriate antibiotic treatment over the first 6 hours links with a 7.6% decrease in survival rate. Azrad et al. described a rapid, easy, and reliable method to identity pathogen from blood cultures in 15 minutes [29]. In common clinical testing, it needs 18 - 48 hours to identify pathogenic microbes, which cause urinary tract infections, through urine culture. It takes an additional 18 - 24 hours to conduct antimicrobial susceptibility testing. Generally speaking, rapid identification is essential to reduce time of diagnosis [29]. Li et al. explored the direct identification method combining MALDI-TOF MS with UF-1000i which only took 1 hour and presented speed, accuracy, and good stability [30].

The direct identification has two basic requirements for clinical specimens: (1) The amount of bacteria should be achieved. The minimum of bacteria placed on the target plate by MALDI-TOF MS is about 1 x 10^4 CFU/mL to obtain an accurate fingerprint, and bacteria need to be enriched before identification. (2) The quality needs to be guaranteed in the clinical specimens. Macromolecular components such as hemoglobin and other protein components in the clinical specimens and organic components such as white blood cells in the body fluids might interfere with bacterial mass spectrum peaks, and pretreatment should be done to remove these interfering factors before direct detection.

Positive blood cultures

Direct identification of positive blood cultures needs that the bacterial concentration is always 1 x 10^7 CFU/mL in culture. So, blood samples require a process of enriching bacteria. The pretreatments of positive blood cultures generally consist of two steps: 1) Isolate bacteria from positive blood culture. Gentle decontaminant (such as sodium dodecyl sulphate, saponin and Tween - 80) is first used to dissolve cells in the blood, and then various processes (centrifugation or wash) are employed to remove other interfering factors, and finally bacteria is purified from positive blood cultures. 2) Extract the proteins from the bacteria. Mixed solvents are the most commonly used to extract proteins for identification. Proteins of medium molecular weight on the surface and proteins of low molecular weight presenting in the core of the bacteria are extracted using formic acid/acetonitrile mixed solvents. Although there are still no standardized pretreatment procedures, commercial kits of positive blood culture pretreatment are available currently which can improve identification score and accuracy. But cost of the kits is relatively high and pretreatment procedures are intricate and time-consuming. In addition, another method basing on acoustic capture has been established to collect bacteria in samples which is fast, accurate, and simple to operate. It is expected to replace the traditional separation method.

Midstream urine

Direct detection of pathogenic bacteria in midstream urine samples requires that the amount of bacteria reaches 1 x 10^5 CFU/mL [31]. Pretreatment procedures of midstream urine samples are relatively simple. Cells are removed by low-speed centrifugation, and bacteria are collected by high-speed centrifugation and then precipitated and washed. Proteins are extracted by formic acid and ethyl acid. After centrifugation at high-speed, 1 μL of each supernatant is coated on the target plate, dried, and tested at room temperature.

Application in the detection of antibiotic resistance

The detection content of antibiotic resistance is divided into three categories: the characteristic spectrum peaks of antibiotic-resistant strains, the change of antibacterial agents under the action of active bacterial enzyme, and the bacterial growth in the presence of antibacterial agents. Each of them has its own advantages and disadvantages [32]. Smith developed a new approach of detecting colistin resistance in *Klebsiella aerogenes* and *Enterobacter species* and it did not need to perform antimicrobial susceptibility testing. It is recommended more often compared to conventional methods requiring approximately twenty-four to forty-eight hours. After culture in medium, the new approach detailed in the research took less than one hour to complete the assays which saved time and prevented inappropriate use of colistin [33]. Cordovana et al. investigated the instant detection of carbapenemase-producing *Bacteroides fragilis*, methicillin-resistant *Staphylococcus aureus*, and KPC-producing *Klebsiella pneumoniae* using the MALDI Biotyper system. Comparison with the routine procedure, the MALDI subtyping approach could detect KPC-producing *K. pneumoniae* within twenty-four hours, methicillin-resistant *S. aureus* within twenty-four hours, and carbapenem-resistant *B. fragilis* within twenty-four to forty-eight hours. The new method could ensure a significantly faster report time and an earlier adoption of proper surveillance measures [34].

Operation procedure, common problems, and solutions of MALDI-TOF MS

Operation procedure of MALDI-TOF MS is simple and has low technical requirements for operators. First, a disposable inoculation ring or a sterile toothpick is used to smear the bacteria or fungi on the detection target to form a uniform film. After adding the matrix and drying, films are detected by the new instrument. Matrix transfers energy in the ionization process and it can absorb a large amount of laser light to reduce damage from direct laser to the sample caused by irradiation. The crystallization of sample molecules and matrix can enhance the absorption capacity. α-Cyano-4-hydroxycinnamic acid (α-CHCA), 2,5-dihydroxybenzoic acid,

Table 1. Analysis and solution of common problems.

Common problem	Analysis and solution
Multiple identification results	The sample is not pure. The pure colony should be selected again. Or the colony should be re-purified and identified
	Reagents and target plates lead to cross contamination. Reagents should be re-prepared and target plates should be cleaned again
No peak/poor quality of fingerprints	Pretreatment is not correct. It should be re-selected according to different bacteria type
	The matrix is not added or the concentration of the matrix is too low. The matrix should be added again and the existence of crystallization precipitation in the matrix should be checked. Or the matrix needs to be ultrasonic re-dissolved and re-prepared
	Coating of bacteria or fungi is too thin or too thick
	Target plate is uneven or scratched. The target plate or target position should be re-selected
	Bacteria strain is aging. Fresh bacteria should be re-cultured
	It may be mucous bacteria. The mucous surface should be brushed off and the colony below is to be picked up
Low identification score	Bacteria or fungi are impure or cross-contaminated. Pure colony should be re-selected or re-purified. Reagents should be re-prepared, and the target plate is re-cleaned
	Mass axis is offset. The MS instrument should be calibrated
	Comparison database is incorrect. A new one should be re-selected
	No reference bacteria strain is in the comparison database. A new database should be self-built

and 3,5-dimethoxy-4-hydroxycinnamic acid are widely used as matrix. As α-CHCA is applicable to peptides and small molecular protein detection, it is the most commonly used matrix in the clinical microbiology laboratory. When the matrix is placed for a long time, the precipitation would increase and the mixture becomes insoluble which needs reconfiguration again. A good quality judgement criteria of the matrix is that pale yellow crystals are formed in a short time after adding the matrix on the target plate. Different types of bacteria have different pretreatment methods which will directly affect the extraction and purification efficiency of bacteria protein. In general, direct coating method is used for common Gram-positive and negative bacteria, formic acid extraction in situ is applied in yeast, ethanol inactivation plus formic acid and acetonitrile extraction is suitable for filamentous fungi, and silicon bead breaking wall plus formic acid and acetonitrile extraction is used for *Mycobacterium*. Impurities might enter into the liquid culture medium, and pretreatments such as removal of impurities and bacteria enrichment should be carried out before identification [35]. Analysis and solution of common problem is seen in Table 1 [36].

Quality control of MALDI-TOF MS

Quality control of MALDI-TOF MS identification is mainly divided into two aspects: the application of quality control strains and the quality evaluation index of mass spectrum fingerprints. Quality control strains are detected on MALDI-TOF MS to assess whether the instrument is in good condition, the reagents used are qualified, the operation process is correct, and the personnel operation is standard. And, finally, the comprehensive credibility of the identification results is evaluated. The internationally recognized standard quality control strains mainly include *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), and *Candida Albicans* (ATCC 90028). Quality evaluation indexes of mass spectrum fingerprints consist of peak signal intensity, signal-to-noise ratio, identification score of bacteria, and fungi quality control strains [37, 38].

Laboratory biosafety

In the process of microorganism identification using MALDI-TOF MS, biosafety protection requirements should be met from sample processing to result reporting, and hazardous chemical management should be executed to protect operators and the environment.

Biological hazard

When identifying microorganisms with MALDI-TOF MS, reception and inoculation of specimens should be performed in the biosafety cabinets conforming with the biosafety requirements of Class II laboratory biosafety. When identifying *Mycobacteria*, biphasic fungi, and highly suspected pathogenic microorganism, the laboratory should use effective bacteria inactivation methods and extract proteins after full inactivation in the biosafety cabinets.

Chemical hazard

Chemical reagents mainly refer to the matrix including α-CHCA, formic acid, trifluoroacetic acid, acetonitrile and so on. The reagents should be used and stored in accordance with the regulations and laboratory workers

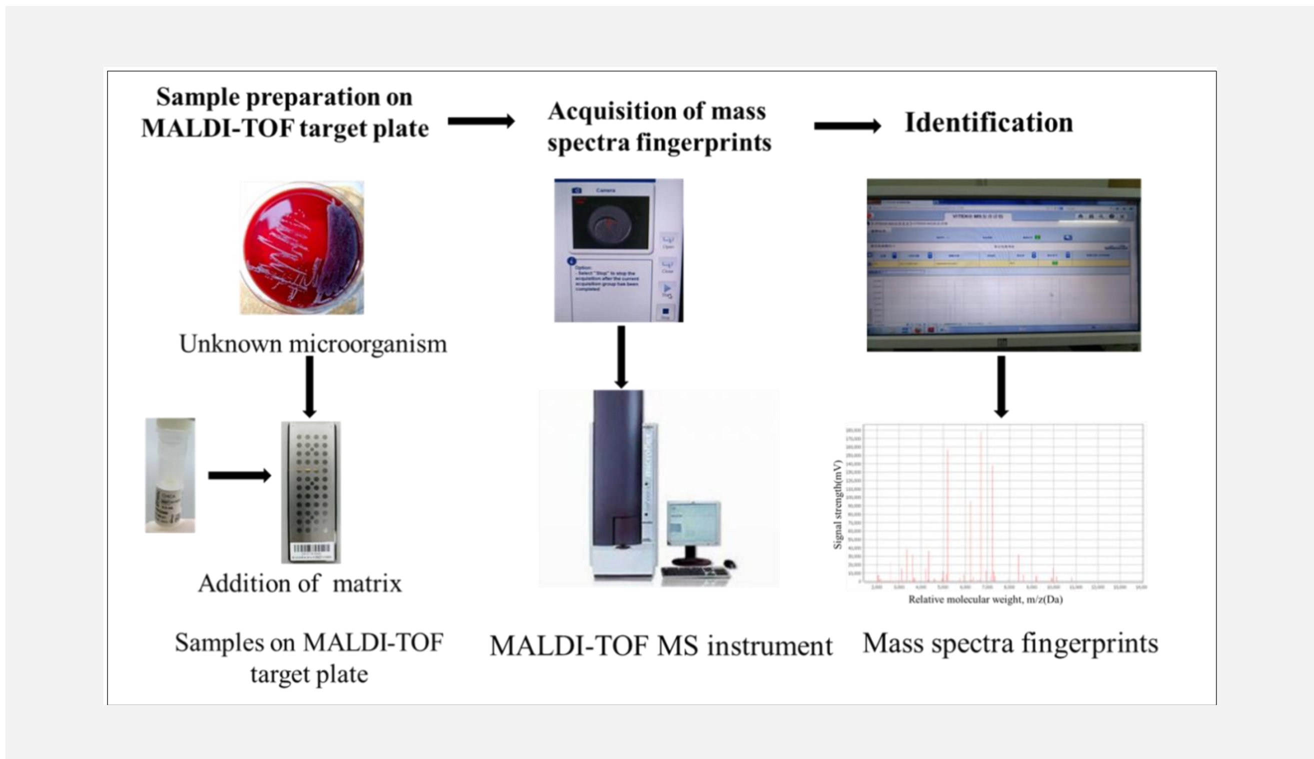

Figure 1. Workflow for microorganism identification with MALDI-TOF MS.

should wear gloves and overall to protect themselves.

RESULTS

Most reviewed studies report the clinical features and applications of MALDI-TOF MS and the internship teaching contents choosing new technology. It is the first report of MALDI-TOF MS technology as an internship teaching content. Primary outcome is that the students understand the theoretical knowledge in detail, master the operation skills of MALDI-TOF MS quickly, and obtain excellent internship performances in the clinical internship through the internship teaching. Secondary outcome is that it is a help to cultivate medical students' train of thought for scientific research and help them understand the application of the new technology in clinical testing and scientific research.

CONCLUSION

With the continuous development of MALDI-TOF MS, it is widely applied in clinical microbiological diagnosis and in scientific research. Laboratory medicine undergraduates should cherish the opportunity to learn the new technology during the internship period and should master basic principles and operation. As internship teachers, it is necessary to introduce it to students during the internship, so that students can quickly master the technology and analyze the detection reports. At the same time, the internship teachers should encourage undergraduates to cultivate creative and innovative thinking in scientific research [39].

To our knowledge, this is the first time to report a relationship between the MALDI-TOF MS technology and internship teaching contents. While preliminary work could offer a theoretical introduction and practical operation of the new technology for undergraduates in laboratory medicine, it has some deficiency. The examinations of both theoretical knowledge and practical operation should be carried out to strengthen the objectivity of graduation practice scores and enhance the motivation of learning. In the future, it will be the direction of efforts to write a clinical or scientific research paper about the new technology into the internship assessment.

Declaration of Interest:
The authors have no conflicts of interest to declare.

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