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Microbial Identification Methods in Pharmaceutical Analysis: Comparison and Evaluation

Sakhno NG[1], Gunar OV[1]

[1]Scientific Centre for Expert Evaluation of Medicinal Products. The Ministry of health of Russia. Petrovsky Boulevard 8, 127051 Moscow, Russia.

Corresponding Author: Sakhno NG, Scientific Centre for Expert Evaluation of Medicinal Products. The Ministry of health of Russia. Petrovsky boulevard 8, 127051 Moscow, Russia, **Tel:** +7-916-561-52-94; **Email:** nadine87@inbox.ru

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ABSTRACT

Rapid and accurate identification of microorganisms is an essential part of pharmaceutical analysis. Contaminants found in ingredients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished products are identified to assist in product investigations. There are number of phenotypic, genotypic and proteotypic methods. A comparison of the database size of representative methods, as well as accuracy of these systems is presented in the review. Emphasis is given to the features of methods and factors affecting the result of identification. Some species of microorganisms within one genus, which determination is problematic from both a genotypic and a phenotypic perspective, are discussed in details. Validation of identification methods is the most important and challenging part of proper automated system choice. Approaches to the evaluation of the system are highlighted in the review.

KEYWORDS: Pharmaceutical analysis; Methods of microbial identification; Database; Accuracy; Validation.

INTRODUCTION

Microorganisms found in the manufacturing environment, water for pharmaceutical use, raw materials and ingredients, intermediates, and finished products are frequently identified to assist in product investigations [1]. The value of the data from an environmental monitoring program is greatly reduced if the microorganisms isolated are not characterized to some degree. Identification of isolates is an essential part of understanding the microbial ecology of a manufacturing facility, monitoring the effectiveness of microbiological control in aseptic environments and investigating of normal microbial populations or sterility failures [2,3].

Routine investigation might include characterization by colony and cellular morphology, gram reaction, and key enzyme activities. This information may be sufficient to confirm that the bacteria found in the sample are typical for that material or manufacturing area or to indicate the effectiveness of environmental control in an aseptic process [1]. The level of identification required in monitoring program depends on the type of the operation and the location from which the isolate was recovered. For example, for non-sterile class D/ ISO 8 manufacturing or ancillary areas it may be sufficient to identify isolates to genus level on a routine basis. But identification of the species is necessary when abnormally high levels of microorganisms are recovered, or for isolates from more critical stages. Microbes isolated from aseptic processing areas often need to be characterized in more detail and may require identification to strain level. Indeed it may be a regulatory requirement to identify isolates to strain level when investigating sterility failures [2].

Microbiological testing of pharmaceuticals may include an identification of colonies found during the total aerobic plate count test. The identification should not merely be limited to the compendia indicator organisms. The importance of identifying all isolates from either or both total plate count testing and enrichment stage will depend upon the product and its intended use. If an oral solid dosage form such as a tablet is tested, it may be acceptable to identify isolates when testing shows high levels. However, for other products such as topical products, inhalants or nasal solutions where there is a major concern for microbiological contamination, isolates from all the parts of analysis should be identified [4]. Microbiological control of cellular products must include both identification of contaminants to a suitable taxonomic level (genus, species) and establishment of an antibiogram [5].

Types of microbial identification methods.

Identification is accomplished by matching characteristics (genotypic or phenotypic) to an established standard (reference) organism such as a type strain [6]. There are number of standard methods for detecting and identifying those indicator pathogens that recommended for quality evaluation of raw materials and finished products by different pharmacopoeia. These procedures include cultivation, morphological and biochemical characterization of isolates [1]. Microbial identification methods can be divided into several groups (Table 1).

Table 1: Types and examples of microbial identification methods [6-8]

Method Types	Examples
Phenotypic methods	Biochemical assays based on physiologica l reactions
	Immunological methods
	Fatty acid profiles
Proteotypic methods	Fourier transform infrared (FTIR) spectroscopy
	Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass-spectrometry
Genotypic methods	Nucleic acid amplification techniques
	Genetic fingerprinting (ribotyping)

Phenotypic methods allow the microbiologist to identify microorganisms to the genus and sometimes to the species level based on a relatively small number of observations and tests [7]. These include biotyping, serotyping, and phage typing.

In biotyping, the biochemical requirements, environmental conditions (pH, temperature, antibiotic resistance, bacteriocins susceptibility) and physiological aspects (colony and cell morphology, cell wall and membrane composition such as fatty acid profile) are investigated [9].

Cultural methods have existed for many years and have been constantly improved. A recent innovation is the appearance of chromogenic substrates which are increasingly used in clinical and food microbiology. The incorporation of such substrates into a selective or non-selective primary isolation medium can eliminate the need for further subculture and biochemical tests to identify certain microorganisms *(i.e. Escherichia coli, coliforms, Salmonella spp., Staphylococcus spp.,* Streptococcus spp. and yeast) and improve discrimination of colonies in mixed culture.

Microorganisms are known to have particular reactions to biochemical substrates, e.g. utilization of specific carbon sources. The identification of culture is done by comparing the biochemical reaction profile with a database, manually or by automated instruments. There are many commercially available kits and analysators such as API® and ID32, BBL™, Crystal™, Biolog Microbial ID System, Vitek 2 Compact, BD Phoenix™ based on such principles. The handling of the system is easy but the interpretation of results can be subjective [5].

Antibody-antigen reactions can be employed to detect unique cellular determinants of specific organisms. These reactions can be linked to agglutination phenomena. Serological typing is one of the oldest immunological-based techniques. It is still important for gram-negative bacteria, such as *Campylobacter spp., E. coli and Salmonella spp.,* and also some gram-positive (for example *Listeria spp.*). Immunoassays not only exist for surface antigens, but also for detection of metabolites, such as toxins. Assays have been described for botulinum, cholera, Staphylococcal enterotoxin, *C. perfringens* enterotoxins, and *B. cereus* enterotoxins.

Bacterial viruses (bacteriophage, phage) can infect host cells causing either lysis or incorporation of their genetic material and expression of novel proteins. These methods can be used in both single and mixed cultures where host specificity allows both detection and identification. These methods are used mainly for research purposes with commercial development aimed principally towards uses in clinical and food microbiology [5,9].

The fatty-acid composition of microorganisms is stable and shows a high degree of affinity within a taxonomic group. The isolate is grown on a standard medium and harvested. The fatty acids are saponified, methylated and extracted. The occurrence and amount of the resulting fatty acid methyl esters are measured by high resolution gas chromatography [5].

In contrast to the examination of the phenotypic characteristic of a microorganism, genotypic techniques study the microbial genome itself [7]. These methods include DNA-DNA hybridization, polymerase chain reaction (PCR), 16s and 23s rRNA gene sequencing, and genetic fingerprinting (ribotyping). Nucleic acid-based methods can be used to screen for specific microorganisms. The steps associated with this activity are sample collection, nucleic acid extraction, target amplification, hybridization, and detection. Genetic fingerprinting is more valuable for strain discrimination than for identification of species [1,5,6].

Third group of methods includes Fourier Transform Infrared (FTIR) spectroscopy, Matrix Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (MALDI-TOF MS). These have not seen widespread use in the pharmaceutical analysis in contradistinction to clinical microbiology. As FTIR spectroscopy and MALDI-TOF MS share some common analytical characteristics, the current convention is to refer to these methods by the alternate description of "Proteotypic" methods [8]. A Fourier transformation of the infrared spectrum of whole microorganisms gives a stable, recognizable pattern

typical of the taxonomic group. MALDI-TOF MS technique is based on the whole bacterial cell analysis. It has yielded unique mass spectra from charged macromolecules from common species of bacteria [1]. This method is applicable both for pure and mixed cultures.

Factors affecting the identification.

The hierarchy of microbial identification errors in descending order of impact is (1) misidentification to genera, (2) misidentification to species, and (3) no identification. Misidentification could lead to inappropriate corrective and preventive actions and product disposition. A microbial identification system may not be able to identify an isolate because the organism is not included in the database, the system parameters are not sufficiently comprehensive to identify the organism, the isolate may be nonreactive in the system, or the species may not have been taxonomically described. Misidentification is difficult to determine, but any microbial identification should be reviewed for reasonableness in terms of the microorganism's morphology, physiological requirements, and source of isolation [6].

Phenotypic methods are limited since microorganisms are capable of suddenly altering their characteristics due to environmental changes or genetic mutations [9]. Expressions of the microbial phenotype, i.e., cell size and shape, sporulation, cellular composition, antigenicity, biochemical activity, sensitivity to antimicrobial agents, etc. frequently depend on the media and growth conditions. Generally, phenotypic methods require a relatively large number of cells in pure, monoclonal culture. Microorganisms isolated from pharmaceuticals and the manufacturing environment may be physiologically stressed and do not grow on routine agar media. Many bacterial species have been found to exist in a viable but non-culturable (VBNC) state which impairs their detection by conventional plate count techniques [6,10]. Furthermore, with fungi, media can affect colony morphology and color, whether particular structures are formed or not etc. [11].

Factors affecting the quality of identification by some biochemical methods are the age of the culture (8- to 24-h cultures are best) and the inoculum optical density [12].

The critical step of many phenotypic identification schemes is determination of Gram reaction. If the wrong characteristic is assigned to an isolate, subsequent testing may be conducted using the wrong microbial identification kit, resulting in an incorrect result [6].

Some bacteria may be Gram-variable. This can be caused by growth stress (e.g., unsuitable nutrients, temperatures, pH, or electrolytes) that results in a number of nonviable, gram-negative cells in a gram-positive culture. However, certain bacteria are notorious for their gram variability even under optimal growth conditions. For example, it is well recognized that Bacillus species and some other gram-positive species (*such as Clostridium spp., Propionibacterium spp., Mycobacterium spp.* etc.) often stain gram-negative or gram-variable as cultures age because of cell wall changes with loss of viability. *Bacillus and Clostridium* species typically are 95% to 100% gram-positive early in their growth phase in broth cultures but become 40% to 50% gram-negative in the late growth phase and 90% to 95% gram-negative in the stationary phase. In the opposite, there are rare instances of classically gram-negative organisms such as *Moraxella and Acinetobacter* species that tend to retain the crystal violet stain and appear to be gram-positive [13-15]. Common pitfalls in this method of staining are that heat fixation may cause Gram-positive cells to stain Gram-negative. In some cases methanol fixation may give more consistent results. Using too much decolorizer could result in a false Gram-negative result, and not using enough decolorizer may yield a false Gram-positive result. To allow identification of errors in staining a Gram-positive and a Gram-negative control should be included in procedure [6].

Genotypic microbial identification methods which are based on nucleic acid analyses may be less subjective, less dependent on the culture conditions, and more reliable because nucleic acid sequences are highly conserved by microbial species. These methods are more technically challenging for the pharmaceutical microbiologist and require more expensive equipment and supplies [1,6]. However, in some cases the use of a sensitive typing technique such as a molecular typing technique or other techniques similar to those used for epidemiological studies (PCR, genotypic, etc.) is obligatory.

For example, a sterility test may be repeated only when it can be demonstrated that the test was invalid for causes unrelated to the product being examined. One of the conditions restricted by the European Pharmacopoeia is demonstration that a microorganism isolated from the product is identical to an isolate from the materials and/or the environment. If tests are performed competently in a clean room environment the chance of simultaneous adventitious contamination occurring in the environment, test sample and negative controls is negligible. But in such case provisions that allow repeat testing based on morphological or biochemical characterization of isolates are not be permitted because samples may contain multiple micro-organisms that are difficult to differentiate without employing sensitive typing techniques [16].

The acceptance and application of nucleic acid amplification methods in routine detection of contaminants has been limited due to the standardization and validation procedures. Many of the aspects that can affect PCR are difficult to control. These are the quality of the DNA template, the environment (humidity, chemical and microbiological cleanliness, temperature), the equipments, personal practice and the reaction conditions and the reaction materials [9,17].

DNA extraction from pharmaceutical samples requires less stringent conditions which might be the result of sample dilution during preenrichment and lack of inhibitory substances such as those as found in food, clinical and

environmental studies [18]. The choice of extraction method can crucially influence the determination of organisms and it is often a trade-off between costs, optimal yield of DNA and removal of substances that could influence the PCR reaction [19].

Comparison of identification systems

An important consideration with a microbial identification technology is the number and types of genera and species in the database and time required to identify the microorganism. A comparison of the database size of representative phenotypic and genotypic microbial identification methods is presented in Table 2.

It should be mentioned that software of some systems (e.g. Vitek, MIDI, MicroSeq, MALDI-Biotyper) allow the user to generate database of frequently encountered isolates, and this is particularly useful for the identification of industrial environmental isolates [20].

There are some species of microorganisms, which determination is problematic from both a genotypic and a phenotypic perspective.

In particular, *Bacillus cereus, B. thuringenisis* and B. anthracis are so closely related that most identification systems cannot distinguish them. The increased recognition of B. cereus pathogenicity and its possibility to cause infections, while contaminating pharmaceutical products, can require improving methods of its identification [21]. The FDA's Bacteriological Analytical Manual (BAM) recommends a series of tests to distinguish among *B. cereus, B. thuringiensis, B. mycoides, B. weihenstephanensis, B. anthracis and B. megaterium* after cultivation on selective and differential media [22,23]. However, results with atypical strains of B. cereus are quite variable, and further testing may be necessary to identify the isolates. A genotypic approach may be more precise and accurate. Using multiple loci comparisons (Multi-Loci Sequence Typing or MLST) together with phylogenetic

Table 2: Comparison of databases and time-to-detection of identification systems.

Method	System	Manufacturer	Database size	Time
Biochemical	API® and ID32	Biomerieux, France	> 600 species of bacteria and yeast	18 – 48 h (2 h – for *Neisseria spp., Haemophillus spp., Moraxella catarrhalis*)
	BBL™ Crystal™	Becton Dickinson, USA	> 400 taxons and 120 genera of clinically important organisms	4 – 24 h
	Biolog Microbial ID System	Biolog Inc., USA	> 2500 species of bacteria, yeast and mold	4 – 26 h
	Vitek 2 Compact	Biomerieux, France	> 450 taxons	2 – 24 h
	BD Phoenix™	Becton Dickinson, USA	> 200 taxons	4 – 24 h
Fatty acid methyl ester analysis	MIDI Sherlock	MIDI, USA	> 2500 species including 700 environmental aerobic, 620 anaerobic microorganisms and 200 yeast	Overnight
MALDI-TOF mass-spectrometry	MALDI-Biotyper	Bruker Daltonik GmbH, Germany	> 2500 species (5600 strains) of microorganisms	Minutes
	Vitek MS	Biomerieux, France	755 clinically important species	Less than 2 minutes
Fourier transform–infrared (FT-IR) spectroscopy	IFS-28B FT-IR spectrometer	Bruker Daltonik GmbH, Germany	730 bacteria strains covering 220 species out of 46 genera, 332 yeast strains covering 74 species out of 18 genera	Minutes
Nucleic acid extraction, PCR amplification and rRNA base sequencing	MicroSeq™ Identification System	Applied biosystems, USA	> 2300 bacteria species, 1100 fungi species	2 – 4 h
Ribotyping	RiboPrinter® System	DuPont Nutrition & Health, USA	> 5700 patterns covering more than 180 genera, 1200 species	8 h

analysis looks promising [21,24].

Another problematic group of microorganisms is fungi, which are classified and identified by their morphological features rather than nutritional and biochemical differences. Fungal taxonomy is complicated the existence of teleomorphs (sexual states) and anamorphs (asexual states) for the same fungus that develop at different times under various nutritional conditions, leading to dual species names [1].

According to most pharmacopoeia (EP 8.0, USP 38, JP XVI, etc.) medicines for vaginal use must be free from *Candida albicans*. However, identification methods are not described and should be chosen by investigator. It is well known that several non-albicans species such as *C.tropicalis, C. dubliniensis,* are phenotypically similar to *C. albicans* and cannot be distinguished using the standard techniques. The crystallographic method allows identifying Candida spp. in 16-18 h. The crystallograms showed characteristic structures in the form of a complex of crystals specific for different species *(C.albicans, C.stellatoidea, C.tropicalis, C.krusei etc.)* [25]. Nucleic acid sequencing provides the differentiation between *C. albicans and C. dubliniensis* [26].

The database of MALDI-TOF MS comprises mostly clinically relevant microorganisms and environmental isolates and it has been successfully applied in yeast and mould identification [27]. However, in the pharmaceutical analysis the cultural technique is still the simplest method of yeast differentiation. CHROMagar is widely used to identify *C. albicans, C. krusei, and C.tropicalis.* This medium contains chromogenic substrates which react with enzymes secreted by the target micro-organisms to yeald colonies of varying colours [28,29].

Fungal contamination have increasingly accounted for medicinal products recalls associated with microbiological incidents (up to 21%). The main types of isolates were speciated as filamentous (moulds) fungi such as *Aspergillus spp., Penicillium spp., Fuzarium spp.,* Rhizopus spp. Moulds are ubiquitous in nature and, therefore they pose a risk to pharmaceutical manufacturing operations. *Aspergillus spp., Cladosporium spp., Penicillium spp., Trychophyton spp.,* and other filamentous fungi have caused significant microbial contamination issues in products and manufacturing area. One of the problems with tracking contamination through a pharmaceutical facility is the lack of species identification performed. To develop the effective corrective action when out of specification results are obtained, accurate fungal identification is needed if the contamination source is to be determined [30]. The characterization of moulds is usually performed using microscopic features (structures bearing spores and spores themselves). The Biolog Filamentous Fungi system employs redox chemistry. Based on reduction of tetrazolium in response to metabolic activity, the reaction occurs in a 96-well plate that allows the analysis of fungal growth via turbidimetric means [1]. The MicroSeq system appears to be accurate and useful for the identification of filamentous fungi. However, the library does not include some of the common species, i.e., *Blastomyces dermatitidis and Coccidioides immitis* and other environmental flora that cause disease in immunocompromised patients [31]. MALDI-TOF MS proved to be a reliable alternative tool for mould identification [32,33]. However, conventional methods could not be simple replaced by this system because of a high rate of nonidentifications (16.5% compared to 4% ones when using the conventional identification algorithm). The use of MALDI-TOF MS in diagnostic mycology for the identification of moulds is limited mainly due to the poor fungal coverage of the commercial databases and the requirement of extended sample preparation to achieve good-quality mass spectra. This procedure including a liquid subculture and the ethanol-formic acid extraction is more time-consuming than the direct transfer preparation protocol that is suitable to identify most bacteria. Collecting fungal material directly from solid medium (agar plates) instead of harvesting it from liquid subculture has been proposed by others and could simplify sample preparation and save time [32].

Staphylococus aureus is one of the objectionable microorganisms in pharmaceuticals. PCR technique supports the quick identification of 1-2 CFU of microorganism to the species level. The minimum detection limit of S.aureus depends on the cultivation conditions. In some cases preenrichment step may be necessary [34]. The combination of Raman spectroscopy with a support vector machines is an extremely capable method of identifying single bacteria Staphylococcus spp. of different cultivation conditions not only on the species level but also on the strain level [35]. However, series of standard cultural and biochemical tests allow to differentiate and identify S.aureus with high level of accuracy which can reach 98.6% [22,36].

Automated microbiological identification systems are based on different analytical techniques, and each has restrictions due to method and/or database limitations and inherent shortcomings in terms of accuracy, reproducibility, technical complexity, rapidity, and cost. The choice must be made regarding the appropriate technology to use in the routine pharmaceutical microbiological testing laboratory with these limits in mind as well as a thought to the need for the level of identification (genus, species, strain) needed for the particular situation [1]. Thus, it is important to define the specific requirements and to purchase the appropriate system to meet those needs. This is also the first step in qualifying the microbial identification technology for use in the lab [8].

Validation of methods for microbial identification.

According to USP Chapter <1113>, the validation of identification system man include one of the following: (1) using an existing system for parallel testing of microbial isolates obtained from routine testing (the number of isolates tested may be as high as 50, and any discrepancies in identification can be arbitrated using a referee method); (2) testing 12–15 known representative stock cultures of different commonly isolated species for a total of 50 tests; or (3) confirming that 20–50 organism identifications, including

15–20 different species, agree with the results of a reference laboratory testing of split sample. With identification systems, verification of the identity of the species should be evaluated and the level of agreement should be considered. The most important verification test is an accuracy, which can be defined as a ratio between number of correct results and total number of results. Other measurements are reproducibility, sensitivity, specificity, and false positive and negative results. Additional instrumentation and method validation activities may also be necessary, as required by the user, and as appropriate for the technology platform representative of the alternative or rapid system. The user should establish suitable acceptance criteria for validation parameters taking into account method capability. However, these criteria should be applied critically, as the results depend on the organisms selected in the evaluation [37].

Traditionally the validation procedure provides the evidence that a new system meets the results of a "gold-standard" method. One of the challenges in microbial identification is that all names are, by their nature, arbitrary and there are no gold standards in common use. In this case, the "correctness" of the result can be defined by authority (i.e., use of ATCC-type strains or in-house strains identified by several different methods consistently) [8,38].

Accuracy of automated identification system ranges widely depending on type of microorganism (Table 3).

Groups of organisms that are challenging to identify (e.g nonfermenting bacteria, corynebacteria, *Staphylococcus spp, B.cereus, fungi*), yielded lower levels of agreement. In average, the accuracy of such methods as ribotyping and MALDI-TOF MS was higher than other ones.

CONCLUSION

To date there are several techniques such as MALDI-TOF MS or ribotyping that seem to be the attractive technologies of rapid microbial identification. The absence of sample preparation, coupled with rapid analysis and high throughput make them indispensable for clinical investigations where precise identification affect diagnosis and treatment options. The ability of MALDI-TOF MS to identify bacteria to the species level in pure cultures and simple microbial mixtures has been established. Besides, this method is free from restrictions related to conditions of microbial cultivation [56].

However, considering the range of factors (safety issues, breadth of the method application, history of regulatory approval, equipment cost, rapidity of the method, number of identification runs per day, number of vendors who can supply the equipment, complexity of the method, ease of validation, training requirements, potential cost savings) phenotypic methods based on carbon utilization and biochemical reactions are still the most widely used in pharmaceutical analysis [1].

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Table 3: The accuracy of some automated identification systems.

Method	System	Accuracy, %	References
Biochemical	BD Phoenix™	75,6 – 96,1	O'Hara [39]; Williams et al. [40]; Donay et al. [41]; van Veen et al. [42]; Funke et al. [43]; Schreckenberger et al. [44]; Eigner et al. [45]
	Vitek 2 Compact	42,5[a] – 94,0	Funke et al. [12];van Veen et al. [42]; Schreckenberger et al. [44]; Eigner et al. [45]; Guo et al. [46]; O'Hara et al. [47]; Odumeru et al. [48]
	Biolog Microbial ID System	47,5[b] – 97,5	Sutton [1]; Odumeru et al. [48]; Tang et al. [49]
Fatty acid methyl ester analysis	MIDI Sherlock	75,0 – 77,8	Tang et al. [49]; Kellog et al. [50]; Morey et al. [51]
MALDI-TOF MS	MALDI-Biotyper / Vitek MS	79,0[c] – 98,7	van Veen et al. [42]; Guo et al. [46]; Schulthess et al. [32]; Becker et al. [33]
PCR amplification and rRNA base sequencing	MicroSeq™ Identification System	55,9[c] – 97,0	Sutton [1]; Hall et al. [31];Drancourt et al. [52]; Tang et al. [49]; Woo et al. [53]; Kim et al. [54]
Ribotyping	RiboPrinter® System	81,0 – 93,8	Sutton [1]; Carretto et al. [55]

[a] – Bacillus cereus; [b] – Staphylococcus aureus; [c] – Filamentous fungi.

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