Microbial identification

9

9.1 Introduction

This chapter addresses some of the methods deployed for achieving species identification of an unknown microorganism (a bacterial or fungal species can be defined as a population of cells with similar characteristics). Hence, identification is the determination of whether a microorganism should be placed within a group of organisms known to fit within a classification scheme. Microbial identification can be defined as "microbial characterization by a limited spectrum of tests pre-chosen and appropriate to the problem being studied" [1].

Classically, microbial identification is undertaken using staining techniques and various agars and tests aimed at differentiating one probable species from another. This process was advanced during the 1970s through the advent of the API (analytical profile index) test strip, consisting of a series of militarized biochemical substrates contained within ampoules. Since the 1990s, a series of semiautomated phenotypic methods became available for the microbiology laboratory in a format that was relatively easy to use and relatively affordable [2]. The 2000s saw an equivalent range of more powerful genotypic methods introduced [3]. These different methods are discussed in this chapter.

9.2 Microbial taxonomy

The objective of microbial identification is to differentiate one microbial isolate from another and then to place that isolate into a family (genus) and a species (which is the best that can be achieved at the phenotypic level of identification) or even as a particular strain (through genotypic identification; a strain is a genetic variant or subtype of a microorganism). The differences between phenotypic and genotypic identification methods are outlined below.

In relation to taxonomy, this relates to the classification of an organism. The main taxonomic terms of importance to microbiology are:

- family: a group of related genera;
- genus: a group of related species;
- species: a group of related strains;
- type: sets of strain within a species (e.g., biotypes, serotypes);
- strain: one line or a single isolate of a particular species.

With an identification result, the most commonly used term is the species name (e.g., *Staphylococcus aureus*). There are always two parts to the species name, one defining the genus in this case "Staphylococcus" and the other the species (in this case "aureus"). Sometimes the species cannot be determined and the result,

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drawing on the same example, would be "*Staphylococcus* species" (commonly the abbreviation "sp." is used in the singular or "spp." in the plural in place of the specific epithet. In this case, the microorganism is written in short-hand as *Staphylococcus* sp.).

9.3 Identification methods

Microbial identification is the determination of whether an organism should be placed within a group of organisms known to fit within some classification scheme. While it is possible for an experienced microbiologist to "identify" a microorganism by its visual appearance on a standard agar, such methods are generally unreliable and are no substitute for a standard identification method.

Identification methods can be divided into two groups: phenotypic and genotypic. The genotype–phenotype distinction is drawn in genetics. "Genotype" is an organism's full hereditary information, even if not expressed. "Phenotype" is an organism's actual observed properties, such as morphology, development or behavior [4]. The phenotype can alter, or at least appear different under varying environmental conditions. For example, a microbial colony may appear a different color on two different culture media.

Phenotypic methods are the most widespread due to their relatively lower costs for many laboratories. It should be recognized, however, that expressions of the microbial phenotype, that is, cell size and shape, sporulation, cellular composition; antigenicity, biochemical activity, and sensitivity to antimicrobial agents frequently depend on the media and growth conditions that have been used. In addition, phenotypic reactions typically incorporate reactions to different chemicals or different biochemical markers. These rely on the more subjective determinations. The reliance upon biochemical reactions and carbon utilization patterns introduces some disadvantages to the achievement of consistent (repeatable and reproducible) identification. To improve on the classical methods of biochemical identification, several developments have been made and refined in recent years. Collectively these methods are considered as modern biochemical identification techniques.

Genotypic methods are not reliant upon the isolation medium or growth characteristics of the microorganism. Genotypic methods have considerably enhanced databases of different types of microorganisms. Genotypic methods have opened up a whole new set of species and subspecies, as well as re-classifying species and related species (thus, taxa are often similarly grouped by phenotypic methods are actually polyphyletic groups, that is they contain organisms with different evolutionary histories which are homologously dissimilar organisms that have been grouped together) [5]. Genotypic methods utilize one of the two alternatives: hybridization or sequencing (most commonly of the gene coding for 16S rRNA (ribosomal ribonucleic acid)). With hybridization, DNA–DNA homology (or how well two strands of DNA from different bacteria bind (hybridize) together) is

used to determine the relatedness of two microorganisms. With sequencing, the reason for methods examining the 16S rRNA region of the genome is:

- It is present in almost all bacteria, often existing as a multi-gene family, or operons;
- It is "highly conserved." The function of the *16S rRNA* gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution);
- The *16S rRNA* gene is large enough for informatics purposes.

For filamentous fungal identification, this requires more expensive methods such as polymer chain reaction (PCR)-based internal transcribed spacer (ITS) regions sequencing by molecular methods. Advances have also been made with newer techniques, such as beta-D-glucandetection (using a (1–3)-β-D-glucanassay, based on the *Limulus* amebocyte lysate test) for the detection of fungal infections.

9.4 Phenotypic methods

Phenotypic methods allow the microbiologist to identify microbial species to the genus and sometimes to the species level based on a relatively small number of observations and tests. These are primarily growth-dependent methods, and identification must begin with a pure culture. The test comprises colony and cell morphology, Gram reaction and other staining characteristics, and metabolic and growth characteristics. The latter sets of tests are commercially available in test kits that are either read manually or through automated readers [6].

Before embarking on an identification test, the microbiologist needs to begin with a pure culture. Starting with a pure culture is the essence of good identification. This means that as a first step for identification is an aseptic sub-cultivation onto a suitable medium (certain media are required for specific microbial identifications systems), followed by incubation at a suitable temperature. Furthermore, with fungi, media will affect colony morphology and color, whether particular structures are formed or not, and may affect whether the fungus will even grow in culture. Therefore, the selection of media is as important as the subculture technique.

9.4.1 Colony and cell morphology

The first step of most identification schemes is to describe the colony and cellular morphology of the microorganism. Colony morphology is normally described by directly observing growth on agar, where the colony will appear as a particular shape (such as raised, crenated (having a scalloped edge), and spherical).

9.4.2 Staining techniques

9.4.2.1 Gram-stain

The primary staining technique used to differentiate bacteria is the Gram stain. The Gram stain is an important tool in the process of bacterial identification; this is through dividing bacteria into two groups (the so-called Gram-positives and Gram-negatives)

and in allowing their morphological types (coccid or rod shaped) to be clearly seen by using a compound light microscope and oil immersion lenses (typically a 100× magnification is used).

The Gram stain method employed is a four-step technique: crystal violet, a triarylmethane dye (primary stain); iodine-potassium compound (mordant); alcohol or acetone (decolorizer); and safranin (counter stain). Carbol fuchsin is sometimes substituted for safranin since it more intensely stains anaerobic bacteria.

Iodine as the mordant means that it is a substance that increases the affinity of the cell for crystal violet so that crystal violet is more difficult to remove from the cell. With the test, Gram-positive organisms retain the crystal violet stain and appear blue; Gram-negative organisms lose the crystal violet stain and contain only the counter-stain safranin and thus appear red [7].

The chemical reaction at play is (Figures 9.1 and 9.2):

- *Step 1*: crystal violet (CV) dissociates in aqueous solutions to form CV+ and chloride (Cl^-) ions. These ions penetrate through the cell wall and cell membrane of both Gram-positive

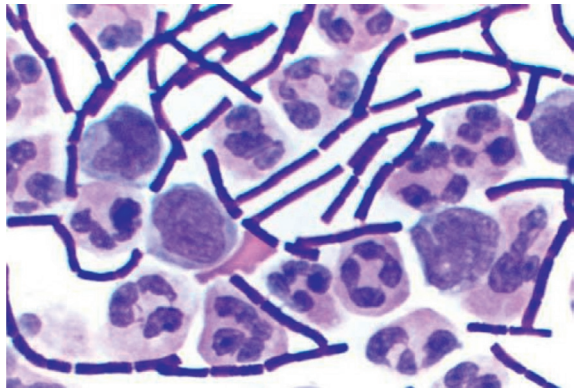

Figure 9.1 A Gram-positive stain, showing a *Bacillus* species.
Image: Creative Commons Library.

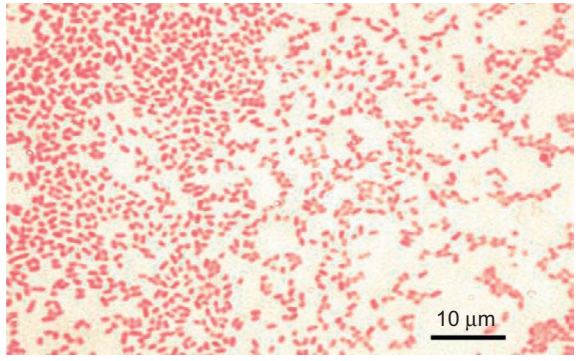

Figure 9.2 A Gram-negative stain, showing a *Pseudomonas* species.
Image: Creative Commons Library.

and Gram-negative bacteria. The CV+ ion interacts with negatively charged components of bacterial cells and stains the cells purple;

- *Step 2*: iodide (I^- or I^{-3}) interacts with CV+ and forms large complexes of crystal violet and iodine (CV–I) within the inner and outer layers of the cell. Iodine acts as a trapping agent in that it prevents the removal of the CV–I complex;
- *Step 3*: when a decolorizer is added, it interacts with the lipids of the cell membrane. A Gram-negative cell loses its outer lipopolysaccharide membrane, and the inner peptidoglycan layer is left exposed. The CV–I complexes are washed from the Gram-negative cell along with the outer membrane.

In contrast, a Gram-positive cell becomes dehydrated from the decolorizer treatment. The large CV–I complexes become trapped within the Gram-positive cell due to the multilayered nature of the peptidoglycan;

- *Step 4*: after decolorization, the Gram-positive cell remains purple, and the Gram-negative cell loses its purple color. A counterstain (such as safranin) is applied to give decolorized Gram-negative bacteria a pink/red color.

Some bacteria, after staining with the Gram stain, yield a gram-variable pattern: a mix of pink and purple cells are seen. This can relate to the age of the culture (which is why cultures subcultured within 24 h work best) or due to the nature of the bacterium (the genera *Actinomyces*, *Arthobacter*, *Corynebacterium*, *Mycobacterium*, and *Propionibacterium* have cell walls that are sensitive to breaking and, thus, some cells can appear "Gram-negative"; alternatively, with *Bacillus*, *Butyrivibrio*, and *Clostridium*, a decrease in peptidoglycan thickness during growth coincides with an increase in the number of cells that stain Gram-negative).

9.4.2.2 Bacterial spore stain

Physiological adaptation into the endospore production is a very important survival characteristic of some Gram-positive rods such as species of *Bacillus* and *Clostridium*. Endospore formation is usually triggered by a lack of nutrients; it is a stripped-down, dormant form to which the bacterium can reduce itself. The endospore consists of the bacterium's deoxyribonucleic acid (DNA), ribosomes, and large amounts of dipicolinic acid.

Spore staining using malachite green (a triarylmethane dye) and a safranin (an azonium compound) counterstain becomes a very useful tool in identifying the presence or absence of spores, and the location of spores such as terminal and subterminal, which may be used as a distinguishing feature in some spore formers. This is referred to as the Schaeffer–Fulton stain.

With this method, using an aseptic technique, bacteria are placed on a slide and heat fixed. The slide is then suspended over a water bath with porous paper over it, so that the slide is steamed. Malachite green is applied to the slide, which can penetrate the tough walls of the endospores, staining them green. After 5 min, the slide is removed from the steam, and the paper towel is removed. After cooling, the slide is rinsed with water for 30 s. The slide is then stained with diluted safranin for 2 min, which stains most other microorganic bodies red or pink. The slide is rinsed again, and blotted dry and examined under a light microscope (Figure 9.3).

There are alternative staining methods, such as the Moeller stain, where carbol fuchsin (a mixture of phenol and basic fuchsin) is the primary stain used in this

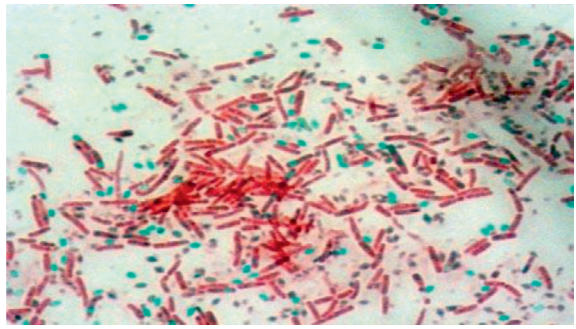

Figure 9.3 An endospore stain, where bacterial rods stain red and bacterial spores stain green. Image: Creative Commons Library.

method. Endospores are stained red, while the counterstain, methylene blue (a heterocyclic aromatic chemical compound) stains the vegetative bacteria blue.

9.4.2.3 Fungal staining

The identification of fungi using macroscopic and microscopic techniques is difficult and requires a trained eye. Lactophenol cotton blue stain is used in wet mounts for microscopically examining yeast and filamentous fungi. The stain serves as both a mounting fluid and stain. Staining the specimen light blue allows subtle features such as septa, special mycelia (hyphae weave together to form mycelium) and spore structures to be easily visualized by microscopy (Figure 9.4).

9.4.2.4 Ziehl–Neelsen stain

The Ziehl–Neelsen stain is a special bacteriological stain used to identify acid-fast organisms, mainly Mycobacteria. The reagents used are Ziehl–Neelsen carbol fuchsin, acid alcohol, and methylene blue. Acid-fast bacteria will be bright red after staining.

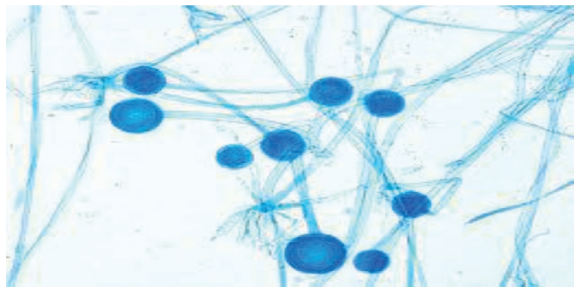

Figure 9.4 A fungal stain, using lactophenol cotton blue.
Image: Creative Commons Library.

Given that, such bacteria are not readily detected within the pharmaceutical environment, and this stain is not discussed further.

9.4.3 Growth based and metabolic tests

Further identification examines the growth and metabolism of the bacterium. Differentiated culture media can be used here. This is media that selectively promotes the growth of certain bacteria. However, due to many variables of growth, such cultural techniques cannot always be assumed to be definitive. It is sounder practice to use a premade identification test kit.

The most common techniques used, based on their costs and long history, are biochemical tests. Biochemical test investigates the enzymatic activities of cells serve as powerful tests in the identification of bacteria. The basis of many biochemical tests is the fact that bacteria are capable of using different carbon sources to obtain the energy needed to sustain life. Which carbon sources react and which do not allows a probabilistic assessment to be made.

An example of biochemical profiling is the API identification system or the alternative BBL-crystal system (microtubes containing dehydrated substrates). The API was the first such identification test and was invented during the early 1970s by Pierre Janin of Analytab Products, Inc. (Figure 9.5).

Many laboratories now adopt semiautomated phenotypic identification systems, such as VITEK (a card preloaded with various biochemical broths) or OmniLog (a miniaturized system utilizing the microtiter plate format). Such phenotypic methods tend to work on the process of elimination. If test A is positive and B is not, then one group of possible microorganisms is included, and another is excluded. From this, tests C and D are performed, and so on. The test results are compared against databases that work on the basis of a dichotomous key [8]. An alternative is the analysis of cellular fatty acids by using gas chromatography (where patterns of fatty acid esters are determined by gas chromatography) [9]. Fatty acid methyl ester analysis by gas chromatography (GC-FAME) has been used for over many years to identify microbes in environmental and clinical settings; however, such systems are less common within the pharmaceutical microbiology laboratory.

More recently developed phenotypic methods include mass spectrometry and flow cytometry. Mass spectrometry can be orientated toward the identification and

Figure 9.5 A 20E API identification test strip.
Image: Tim Sandle.

classification of microorganisms by using protein "fingerprints" (characteristic protein expression patterns that are stored and used as specific biomarker proteins for cross-matching). When identifying bacteria with a device like a Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) instrument, a single isolated colony or simple cell extract is spotted onto a stainless steel target plate and overlaid with an ultraviolet absorbing molecule. The target plate is inserted into the MALDI-TOF. Nitrogen-pulsed laser ionization is then applied to the sample, and the proteins are ionized. They are separated based on their mass/charge ratio. The resulting spectra, a protein fingerprint (which falls within the 2000–20,000 Da range), are compared with a database of known spectra.

Flow cytometry is a technique that employs serological methods to analyze cells suspended in a liquid medium by light, electrical conductivity or fluorescence as the cells individually pass through a small orifice. The use of fluorescent stains or fluorogenic substrates in combination with flow cytometry methods allows the detection and discrimination of viable culturable, viable nonculturable, and nonviable organisms [10].

9.5 Genotypic methods

Genotypic techniques study the microbial genome and, unlike phenotypic methods, they are not reliant upon the isolation medium or growth characteristics of the microorganism.

In bacteria, there are three genes that make up the rRNA functionality, these are: 5S, 16S, and 23S rRNA. Of these, the *16S rRNA* gene is most commonly used to identify the species. The 16S (small subunit) rRNA gene is selected for a number of reasons: (i) it is present in all organisms and performs the same function; (ii) its sequence is sufficiently conserved and contains regions of conserved, variable and hypervariable sequence; (iii) it is of sufficient size (around 1500 bases) to be relatively easily sequenced but large enough to contain sufficient information for identification and phylogenetic analysis (Figure 9.6).

An example of this technology is the RioPrinter (manufactured by Dupont Qualicon). This is an automated Southern blot device that uses a labeled ssDNA probe from the 16sRNA codon. The RiboPrinter uses a restriction enzyme, and strains can be identified and/or characterized by analyzing the ribosomal DNA banding pattern. Every time a sample is run, the RiboPrinter system produces an exact genetic snapshot of the microorganism that is linked to historical data. This genetic snapshot is akin to a "fingerprint." The DNA fingerprint is generated from regions of the rRNA genes (5S, 16S, 23S, and the spacer region including Glu-tRNA) that is unique to the microorganism at the strain level.

Another rapid method is a PCR system that uses a form of "bacterial barcodes" where the amplified genetic sequence is separated by gel electrophoresis and visualized to give a "barcode" specific to that strain. PCR is a technique which uses a DNA polymerase enzyme to make a huge number of copies of virtually any given piece of DNA or gene. It facilitates a short stretch of DNA (usually fewer than 3000 "base

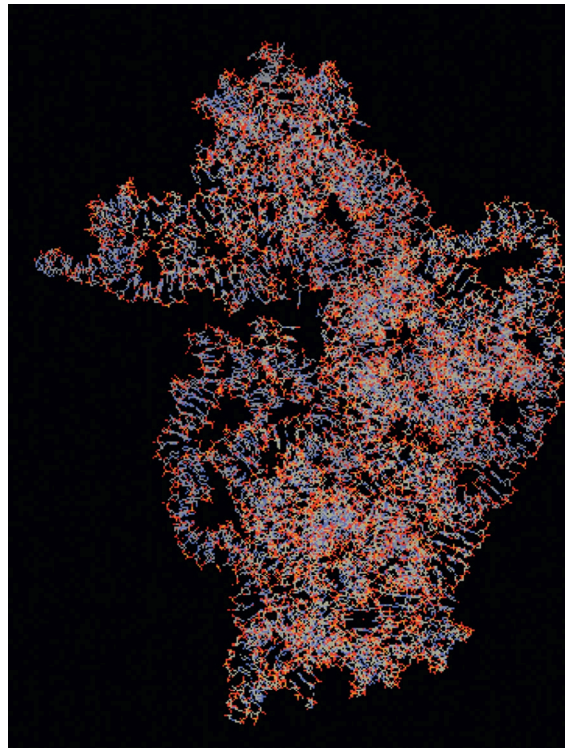

Figure 9.6 A representation of 16s rRNA.
Image: Creative Commons Library.

pairs") to be amplified by about one million-fold. With this comparative test, differences in the DNA base sequences between different organisms can be determined quantitatively, such that a phylogenetic tree can be constructed to illustrate probable evolutionary relatedness between the organisms [11]. An example of such a system is the MicroSeq manufactured by Applied Biosystems.

A final genotypic method is the Bacterial Barcodes system (DiversiLab). This system is also based on the PCR technology, using as a primer a sequence homologous to a repetitive sequence in the bacterial genome. The amplified sequence is then separated by gel electrophoresis and visualized to give the "barcode" specific to that strain.

9.6 Method validation

It is important that when microbiological identification methods are introduced into a laboratory that they are validated or verified. To begin with, if an automated

instrument is purchased, this requires qualifying. Validation involves a series of steps, which can be summarized as:

1. Installation qualification: this is the documented evidence that the equipment and associated systems, such as software, hardware, and utilities, are properly installed, and relevant documentation is checked. Documentation may include manuals, certificates, procedures, and calibration records.
2. Operational qualification: this verifies that the system or subsystem performs as intended throughout all anticipated operating ranges and documents the information.
3. Performance qualification: this proves the system performs consistently as intended during normal operational use and remains in compliance with regulatory and user expectations or requirements. Performance of automated microbial identification system is very elaborate and time consuming due to multiple factors such as choice of isolates, operator variability, and the reproducibility of the system itself.

Following validation, or with nonautomated systems, verification of the test is required in order to show that it is suitable. Verification typically consists of [12]:

(a) parallel testing with approximately 50 microbial isolates using an existing system;
(b) the testing of 12–15 representative stock cultures of commonly isolates species (ensuring that these are of a broad enough range to cover the majority of the instruments test array). Here type strains should ideally be used;
(c) confirming that 20–50 microbial identifications, including 15–20 different species, agree with the results of a reference laboratory testing split sample.

The key criteria to be assessed are [13]:

(a) accuracy, which is expressed as a percentage of the number of correct results divided by the number of obtained results, multiplied by 100. To undertake this, type cultures from an approved culture collection should be used;
(b) reproducibility, which is similarly expressed as a percentage. Here the number of correct results in agreement is divided by the total number of results multiplied by 100;
(c) precision, which is achieved by testing multiple samples;
(d) ruggedness, which is undertaken by examining the same cultures with different reagents;
(e) robustness, which can be achieved through using cultures of different ages.

9.7 Conclusion

This chapter has outlined some of the microbial identification techniques undertaken. The techniques described have been divided between phenotypic and genotypic methods. It is important to note that groupings established by phenetic and phylogenetic systems do not always agree and within each grouping the methodological differences and varying contents of different databases will sometimes lead to conflicting analyses.

It is additionally important to understand that any systems used to identify bacteria, whether phenotypic or genotypic, will have limitations, because no single test methodology will provide results that are 100% accurate.

In terms of selecting between methods, this will depend on costs and resources, the time that the microbiologist is prepared to wait for and what level of identification

is required. Some microbiologists are of the view that the only way to characterize a microorganism correctly is through a "polyphasic approach" that is a combination of phenotypic testing methods and genotypic testing methods. This is, however, far too time consuming and too prohibitively expensive for standard laboratories. Most routine testing laboratories select phenotypic test kits and use established contract test facilities where genotypic testing is required.

What is important, when making a selection, is to go back to basics and consider: what is the purpose of the identification? what does the microbiologist need to know? and what does the result tell the microbiologist? These questions can help with selecting and implementing the appropriate microbial identification test.

References

[1] Stackebrandt E. Taxonomic considerations. In: Lewin RA, editor. Prochloron: a microbial enigma. USA: Chapman and Hall; 1989. p. 65–9.
[2] O'Hara CM. Manual and automated instrumentation for identification of enterobacteriaceae and other aerobic Gram-negative bacilli. Clin Microbiol Rev 2005;18(1):147–62.
[3] PDA. Evaluation, validation and implementation of new microbiological testing methods. Technical report no. 33. Parenteral Drug Association, 54(3), Supplement TR33; 2000.
[4] Williams KP, Gillespie JJ, Sobral BWS, Nordberg EK, Snyder EE, Shallom JM, et al. Phylogeny of gammaproteobacteria. J Bacteriol 2010;192(9):2305–14.
[5] Muller M, Davey H. Recent advances in the analysis of individual microbial cells. Cytometry A 2009;75(2):83–5.
[6] Sutton SVW, Cundell AM. Microbial identification in the pharmaceutical industry. Pharmacopeial Forum 2004;35(5):1884–94.
[7] Cowan ST, Steel KJ. Manual for the identification of medical bacteria. Cambridge, UK: Cambridge University Press; 1965.
[8] Sandle T, Skinner K, Sandle J, Gebala B, Kothandaraman P. Evaluation of the GEN III OmniLog® ID system microbial identification system for the profiling of cleanroom bacteria. Eur J Parenter Pharm Sci 2013;18(2):44–50.
[9] Fung DYC. Rapid methods and automation in microbiology. Compr Rev Food Sci Food Saf 2002;1:3–22.
[10] Attfield P, Gunasekera T, Boyd A, Deere D, Veal D. Application of flow cytometry to microbiology of food and beverage industries. Australas Biotechnol 1999;9:159–66.
[11] Kolbert CP, Persing DH. Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Curr Opin Microbiol 1999;2:299–305.
[12] Sandle T. Automated microbial identifications: a comparison of USP and EP approaches. Am Pharm Rev 2013;16(4):56–61.
[13] Green S, Randell C. Rapid microbiological methods explained. In: Halls N, editor. Microbiology and contamination control in pharmaceutical cleanrooms. Boca Raton, FL: CRC Press; 2004. p. 157–80.