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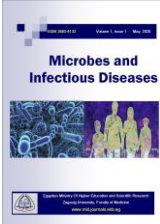

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Review article

Pharmaceutical products microbial contamination: approaches of detection and avoidance

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ABSTRACT

Background: Pharmaceutical products' microbial contamination is a serious worry in the pharmaceutical industry, as it can posture momentous health jeopardies to patients. Contamination with microbes can befall at any stage of manufacturing, starting with raw materials and ending with the finished products. It can also result from a variety of sources counting the environment, working personnel and equipment used. Common microorganisms that contaminate pharmaceutical products include bacteria, fungi, and viruses. The presence of these contaminants can compromise the quality, safety, and efficacy of pharmaceutical products, leading to product recalls, loss of reputation, and financial losses. Therefore, it is essential for pharmaceutical manufacturers to implement robust quality control measures to detect and prevent microbial contamination, including stringent cleaning and sanitation procedures, environmental monitoring, and microbiological testing. In this review, we discussed the main conventional and advanced technologies of detecting microbial contamination in pharmaceutical products. These techniques range from conventional culture methods to the more sophisticated technologies of gene sequencing and spectroscopy.

Introduction

The quality of any pharmaceutical or cosmetic products is dramatically affected by their microbial load [1]. Microbial contamination can lead to undesirable changes in the physical features of the pharmaceutical formulations such as the separation of emulsion into phases, changing the texture of creams, spoilage of syrups with turbidity or deposit formation, in addition to the off changes in odor, colour and taste [2]. These changes are objectionable because of their effects not only on the aesthetic appearance of the products but also due to its effect on the therapeutic outcomes and drug delivery pattern harboring a latent health threat to the consumers [3]. This health hazard is brought about either by the microbial infection itself or by the microbial metabolites and toxins causing symptoms of diarrhea, gastroenteritis and/or abdominal pain of variable severity [4]. Microbes commonly reported to be accused of causing contamination of pharmaceutical products include species of *Erwinia*, *Pseudomonas*, *Lactobacillus*, *Bacillus*, or *Streptococcus* and the genus *Aspergillus* depending on the source of the raw materials and the process of manufacturing and handling. Therefore, owners of the manufacturing authorizations are

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required to test their products and make sure they do comply with the criteria of the marketing authorization to avoid jeopardizing patients' health. In order to objectively achieve these criteria, all stages of drugs manufacturing, including sterile and non-sterile products, are required to be controlled counting raw materials, process of manufacturing and assessment of the end products [5]. A number of techniques have been employed to assess the quality of the pharmaceuticals such as culture-based method, adenosine triphosphate [ATP] bioluminescence and polymerase chain reaction [PCR]. Also introduction of preservatives into the formulated pharmaceutical and cosmetic products have been shown effective in controlling microbial contamination. However, resistance to these preservatives has been documented [6]. In this review, we summarized the technologies employed for pharmaceutical products' microbial contamination detection, methods of prevention and briefly discussing how microorganisms can develop resistance to preservatives.

Detection methods of pharmaceutical products' contamination

Conventional standard microbiological methods, including traditional pharmacopeial methods and simplified commercially available biochemical test kits like the API (analytical profile index) [7,8], are still regularly employed for testing pharmaceutical products and detecting bacterial contaminants. Molecular technologies have recently made a significant impact on pharmaceutical microbiology, providing rapid quantitative and qualitative information on microorganisms present in a given pharmaceutical sample [9,10]. The goal is to achieve more sensitive, precise, and faster microbial detection. Quick microbiological methods are essential for more efficient and prompt control of raw materials and final products They contribute improving reactivity throughout the manufacturing process. Although traditional systems are still widely used in many microbiological experimentations due to their simplicity and cost-effectiveness, they typically require an incubation period of 2 to 7 days on liquid or solid culture media before obtaining the final results of microbial contamination [11,12].

a- Culture-based method

The conventional approach for detecting pathogenic bacteria involves cultivation procedures in a laboratory, a method that, while relatively inexpensive, has several limitations. This method provides information about the diversity and quantity of microorganisms present in a sample, but it is time-consuming and requires significant effort. The process includes preparing and diluting samples, plating and incubating them, counting the resulting colonies, and then characterizing the isolated bacteria, which can take several days. In addition, false positive results are common, especially when working with similar microbial species [13,14]. Another limitation is that culture-based methods are unable to detect non-culturable cells, restricting their usefulness in certain situations. Several attempts have been made to simplify and partially automate the standard method. For instance, the mechanical spreading of the sample on the surface of a pre-prepared agar plate [15], as well as the use of membranes to capture microorganisms before applying them to standard plate culture, have been explored [16]. Additionally, there are methods based on nutrient films or strips that have been developed as substitutes for conducting viable cell counting in the pharmaceutical industry [17,18]. Cultural techniques, despite their limitations, are still commonly used in pharmaceutical analysis for yeast differentiation. CHROM agar remains a popular choice for identifying *Candida albicans, C. krusei* and *C. tropicalis.*

b- ATP bioluminescence

Adenosine triphosphate bioluminescence is a technique employed to measure the amount of adenosine triphosphate [ATP] present in a sample. During the ATP bioluminescence test, the luciferase-luciferin enzyme reacts with the ATP molecule, causing the energy released during ATP hydrolysis to emit a light photon. The luminometer automatically detects and measures the emitted light, providing a quantitative measurement of the ATP present in the sample [19]. Certainly, ATP is present in every living organism and serves as a reliable indicator of cell viability and contamination. Hence, the use of ATP-luminescence technology to detect ATP is preferred over conventional methods, considerably reducing the time required for detection without compromising accuracy [20,21].

Connolly studied rapid microbiological methods, including impedance. The use of impedance ATP bioluminescence techniques has been explored for their potential applicability in pharmaceutical and cosmetic products. Various

substances, such as dextrose, potassium dihydrogen phosphate, magnesium phosphate heptahydrate and ammonium sulphate have been tested against *S. aureus, P. aeruginosa, A. niger* and *C. albicans.* Results have shown a positive association between these techniques and the total bacterial counts for untreated suspensions of *S. aureus, P. aeruginosa* and *C. albicans* [22].

c- Rapid microbiological methods [RMM]

1- Polymerase Chain Reaction [PCR]

It is a highly sensitive method for detecting microbial pathogens in clinical samples, such as *S. aureus, S. epidermidis*, *Enterococcus faecalis,* and *Streptococcus pyogenes.* Real-time PCR, utilising fluorescent signals to monitor PCR products, is a common and reliable technique due to its exceptional sensitivity and specificity [23,24]. PCR operates by amplifying mark DNA and subsequently detecting the amplified products through methods such as electrophoresis, fluorescent labelled antibody probes, dyes, or molecular beacons. Multiplexing, an approach enabling the screening of multiple targets within the same sample, can be employed effectively [17,25,26]. Particularly, Reverse transcription polymerase chain reaction [RT-PCR] is valuable for detecting low concentrations of nucleic acids in a mixtures.

In a study [27] performed by a group of scientists in order to compare multiplex PCR with the conventional culture method, 70 raw materials and finished products were examined for potential microbial contamination. The comparison involved utilizing both conventional culture and PCR methods to identify indicator pathogens. Their result revealed that out of the 70 possible contaminants, only 15 were positive when using conventional culture, while 25 were positive when using the advanced PCR technique. Notably, when samples were artificially contaminated with less than 2 Colony Forming Units per gram [CFU/g], conventional detection methods proved inadequate, whereas multiplex PCR successfully detected concentrations as low as 1 CFU/g or mL. This study highlights the importance of developing multiplex PCR, especially for large-scale pharmaceutical testing scenarios where precise and sensitive assessment and time are perilous. Moreover, it demonstrates the heightened sensitivity of multiplex PCR in detecting each indicator pathogen among the potential contaminants. Among the 70 samples, species-specific multiplex PCR detected *E. coli* in 11%, *S. aureus* in 11%, *P. aeruginosa* in 8%, and *Salmonella* spp. in 4% compared to the detection rates of 1%, 7%, 4% and 1%, respectively, using the conventional culture method.

2- Immunoassay

For many years, immunological tests employing antigen and antibody reactions have been used in medical and pharmaceutical microbiology to detect microorganisms. The enzyme-linked immunoassay [ELISA] test stands out as the most commonly utilised immune-based method for this purpose. In order to attain a measurable concentration of the target pathogen, an overnight incubation is necessary for ELISA tests. This method is routinely hired for the recognition of bacterial species of *Salmonella, E. coli* and *Listeria monocytogenes*. Recent advancements have directed to the advance of entirely automatic ELISA systems capable of conducting tests within a relatively short time frame-ranging from 45 minutes to a maximum of 2 hours, following an overnight incubation [17,18,28]. Various versions of ELISA can be used to detect either antigen or antibody [direct or indirect technique]. This versatility allows for the simultaneous identification of diverse microbial strains. Although ELISA can provide results slightly faster than alternative techniques of plating, culturing, and PCR, it has a high false positive rate and intricate experimental procedures, limiting its widespread application [29,30].

3- Mass spectrometry [MS]

The Matrix-Assisted Laser Desorption/ Ionization [MALDI]-Time-of-Flight [TOF] is a cutting-edge technology for the rapid identification and classification of microorganisms. It operates by using short laser pulses to ionise microbial cells and then accelerating the particles through an electric field within a vacuum system [31,32]. This ionisation process generates a unique spectra profile or molecular fingerprint specific to each microorganism, which is then compared to a pre-existing database for automated identification. In order to make samples for MALDI-TOF MS, a bulky excess of matrix, typically an ultraviolet [UV] absorbing organic acid, is used to crystallise the microorganisms on target plates [33]. The MALDI-TOF MS database primarily includes clinically relevant microorganisms and environmental isolates, and it has a proven success in identifying yeast and mold species [34]. Schematic presentation

of MALDI-TOF MS principle is shown in **figure [1]**.

d- Promising methods for future success

1- Fourier transform near infrared spectroscopy [FT-NIRS]

Fourier transform near infrared spectroscopy **[Figure 1]** joint with chemometric approaches is often selected analytical technology for the rapid measurement of solids, liquids and various pharmaceutical formulae [35]. This approach offers notable advantages, including speed, affordability, and non-destructive properties [36]. Although this technique has been previously applied to detect and identify bacteria in simple liquid suspensions [37], its potential for identifying and quantifying bacteria in pharmaceutical products has not been thoroughly investigated. The method is based on the principles of light absorption and scattering by molecules in the sample. By shining a beam of light in the near infrared region on a sample, the molecules in the sample absorb and scatter some of the light, which generates a spectrum suitable for analysis.

A study was conducted to evaluate the effectiveness of FT-NIRS in identifying and quantifying bacterial contaminants in pharmaceutical preparations and saline solutions [0.9% NaCl]. The researchers utilised five species of bacteria commonly associated with microbial contamination in pharmaceutical products: *Bacillus subtilis*, *E. coli, Pseudomonas fluorescens*, *S. enterica,* and *S. epidermidis*. The methodology was initially validated in buffer solutions and subsequently applied to three distinct pharmaceutical formulations, which were cough syrup, contact lens solution and topical anti-inflammatory solution. They verified the aptitude of FT-NIRS to sense, discriminate and count microbiological contaminations in buffer solutions [Sodium Chloride 0.9%]. Similarly, FT-NIRS exhibited the ability to distinguish uncontaminated pharmaceutical preparations from the contaminated ones for each bacterial species autonomously of its concentration. For instance, it was also conceivable to single out cough syrup solutions that were contaminated with *E. coli* from those contaminated with *S. enterica* and the topical anti-inflammatory solutions contaminated with *P. fluorescens* from those contaminated with *S. epidermidis*. In light of this, it is concluded that infrared based spectroscopic technique offers a substantial potential to be employed as an effective alternative system for quality control in pharmaceutical manufacturing [38].

2- Raman spectroscopy

Raman spectroscopy [RS] **[Figure 1]** is a non-intrusive technique that utilises monochromatic light to interact with chemical bonds in a sample, causing inelastic scattering. This interaction results in a unique Raman spectrum, or "fingerprint" for each molecule based on its chemical and biophysical properties [39]. Through the integration of RS with chemometrics, the pharmaceutical industry has been able to take advantage of its lower cost, quicker quantitative analysis and real-time monitoring capabilities for processes involving molecular changes and chemical environments [40,41]. RS has also demonstrated impressive performance in detecting microbial contamination, with the ability to identify a range of bacterial molecules such as lipids, proteins and nucleic acids [42]. In fact, RS is even capable of accurately distinguishing between various strains of bacteria in water-based preparations and solid medications [43,44]. A study conducted by a research-team in Denmark aimed to evaluate a quick not invasive approach to distinguish pharmaceutical vials contaminated with low concentrations of bacterial strains from sterile vials using dispersive RS alongside with partial least squares-discriminant analysis [PLS-DA]. The RS-PLS-DA technique was tested to detect bacterial species suspended in drug products within their primary packages without the need for further processing and treatment. Three different bacterial species, including sporulated *B. subtilis,* were distinguished without breaking the drug product vial at a low concentration of 10 CFU/ml, even in the existence of other organic molecules in the intact drug product primary container. These results support the use of RS as an auspicious biotechnological tool appropriate for bioburden testing in pharmaceutical industry quality control [45].

Prevention of microbial contamination of pharmaceutical products

One of the main challenges in pharmaceutical production is the control of potential contamination. In order to achieve this, an understanding of the microbial entry points and their different routes is essential [46, 47].

Raw materials

Many consumers assert that microbiological controls are unnecessary for Active Pharmaceutical Ingredients [APIs] and excipients, contending that these substances undergo chemical processing, rendering microbial contamination concerns irrelevant. A variety of APIs and excipients are actually substances that do not allow microbial development, yet under the correct circumstances, others may be susceptible under specific conditions. Because API and excipient manufacturers do not manufacture sterile products, there is a general believe that no sanitation and environmental control are required [48].

Air in the manufacturing area

The atmosphere contains billions of suspending particles and microbes, like *penicillium, aspergillus*, etc., which pose a potential risk of contaminating pharmaceutical products. To prevent this risk, manufacturing processes are conducted in clean rooms and aseptic rooms equipped with a continuous flow of sterile air. The Heating, Ventilation and Air Conditioning [HVAC] system's primary goal is to supply the processing areas with clean air. It is necessary to design the HVAC system to deliver particulate-free and uncontaminated air. Most systems have pre-filters that are >95% efficient and final or terminal filters that are >99.9% efficient [HEPA]. HVAC systems are used in the aseptic areas and single-pass air through the system is essential. Consequently, the air supply should not be recirculated by the system. The air supply should be made up entirely of fresh air and hence cross-contamination is avoided in this way [49].

Equipment

In order to make sterilization easier, equipment for aseptic processing should be properly constructed. The design of the equipment should allow for easy assembly and disassembly, simplifying cleaning, sanitation, and sterilization procedures. The accessibility of the sterilizing agent, the slope of the pipework, and the adequate condensate removal should all be considered when designing fixed equipment [such as large mixing tanks]. Moreover, the impact of equipment design on the clean room environment should also be taken into consideration. For example, it is best to avoid horizontal surfaces and ledges that collect particles. Equipment shouldn't hinder airflow, and in critical areas its design shouldn't interfere with unidirectional airflow [49].

Personnel

Each individual involved in the manufacturing, processing, packaging, or handling of a drug product should have training, education, and experience to make it possible for that person to carry out the required tasks. Personnel involved in the manufacturing, processing, packaging, or handling of pharmaceutical products must wear suitable clothes for the tasks they conduct. To prevent contamination of drug items, protective clothing such as face and head, hand and arm coverings must be worn as compulsory. Additionally, personnel are expected to adhere to stringent hygiene and health practices. To protect the product from contamination by the workers, these types of regulations are especially crucial when aseptic manufacturing is taking place [50].

Water

Water must be readily available for microorganisms to proliferate and aqueous formulations provide quick entree to water for microorganisms. Effects of water in aqueous preparations can be minimized by adding sugar or poly ethylene glycol [PEG] at a high concentration through drying. A high humidity environment during storage can cause water films to develop on the surface of dry products like tablets and bulk oil, which can lead to fungus growth [51,52].

pH

The potential of hydrogen [pH] level has a pivotal role in preventing of microbial attacks. Spoiling is rare at pH levels greater than 8, typically associated with soap-based emulsions. Yeasts or molds are more likely to attack products with extremely low pH values [between 3-4] such as citrus fruit liquid extract, syrups and flavored or un-medicated syrups. By creating organic acid metabolites, yeast can increase the pH of the product and may also promote bacterial secondary growth [53,54].

Storage temperature

Microbial development and deterioration can be effectively controlled through the storage temperature management. Between 20 °C and 60 °C, spoiling is possible while in both low and high temperatures, microbial growth and spoiling are reduced. Products stored in a cold environment, specifically within the range of 8 to 12 °C, may have minimal deterioration. Before filling and sealing, injection water is stored at temperature exceeding 80 °C [55,56].

Packaging and design

Effective packaging is essential to prevent the infiltration of microorganisms into pharmaceutical products during storage or customer usage. Furthermore, packaging has an impact on the stability of pharmaceutical preparations during their shelf life. For example, self-sealing wads are used in multi-dose injection containers to prevent microbial contamination of the product. Then again, wide-mouthed cream jar containers were substituted with narrow nozzles and flexible screw-capped tubes to attain the maximum protection against microorganisms entry [57].

Preservative use in pharmaceutical products

Pharmacists have been aware of the necessity to safeguard their products against microbial contamination for a number of decades, but the science of preservation has only recently received significant consideration. Preservatives are frequently utilised as additives in cosmetics, food and medicinal items, especially in liquid solutions vulnerable to microbial infection. Preservatives are employed to safeguard these preparations, preventing product degradation and modification [58]. A preservative is a natural or artificial chemical that is added to a variety of items to assist stop microbial decay. A natural or artificial substance applied to a pharmaceutical or cosmetic preparation serves as a preservative to prevent spoiling brought on by microbial development [59]. For oral preparations, sodium benzoate, benzoic acid, sodium and potassium, sorbic acid, calcium lactate, paraben [methyl-, ethyl-, propyl] are commonly used as preservatives [60]. Cetrimide, benzoic acid, thimerosal, imidurea, phenyl salicylate, chlorhexidine, benzalkonium chloride, ethylenediaminetetraacetic acid [EDTA], chlorocresol are generally employed as preservatives in topical formulations [61]. Nasal pharmaceutical formulations often require chlorobutanol, EDTA, and benzalkonium chloride as preservatives [62]. Parenteral forms are preserved using parabens [methyl-, ethyl-, propyl-, butyl-, and their combinations], chlorhexidine, formaldehyde, benzyl alcohol, and thimerosal [63]. Ophthalmic preparations typically contain EDTA, benzalkonium chloride, and benzoic acid as preservatives [64].

Resistance

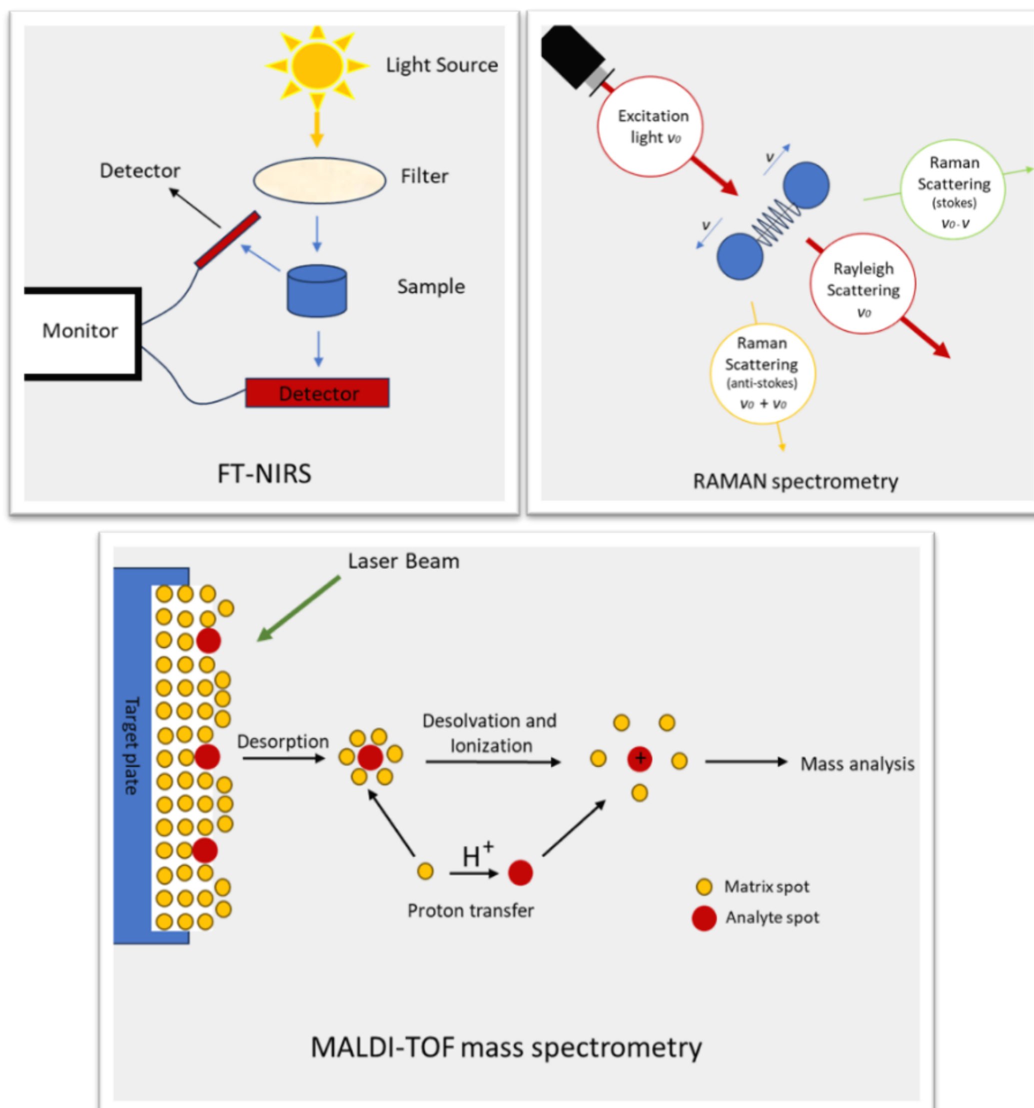
To deeply understand the preservative resistance mechanism, numerous investigations have been conducted. **Russell**'s work provided a substantial insight about the innate defense mechanisms that microbes evolve against preservative substances. It was emphasised that a number of microbial strains have the ability to destroy preservatives with the assistance of certain enzymes. **Park** investigated the initiation of resistance in *S. typhimurium*. The studied microbe encounters extreme stress in its natural niche, such as low pH of the stomach and the hydrophobic weak acids of the intestine. Microorganisms have extensive defense mechanisms to deal with stress and thrive across diverse pH conditions. In a study conducted by **Lin *et al.***, a potent acid was shown to promote resistance in the bacterium *E. coli*. It was discovered that this bacterium was also resistant to benzoic acid. In an additional investigation by **Davis et al.**, *Listeria monocytogenes* was shown to express an increased tolerance to the low pH of 3.0 after it was subjected to a weak acid at pH 5.0. Both instances give insight into the numerous circumstances that can lead to microorganisms developing preservative resistance. Thus, how cosmetics are handled becomes crucial. Any bacterium that is exposed to pH settings that are either mildly or severely acidic could develop a resistant strain and lead to microbial unsteadiness of the pharmaceutical preparation [65,66, 67].

Table 1. Differences between detection methods by their accuracy, time to detect, cost and application in pharmaceutical industry

Detection method	Accuracy %	Time to detect	Cost	Application in pharmaceutical industry
Culture method	50-90%	Days to weeks	Low	Widely used for identifying and quantifying microorganisms, but can be time-consuming and may miss certain species
PCR	>90%	Hours to days	Moderate	Rapid and sensitive detection of specific microbial DNA or RNA sequences. Useful for identifying pathogens or monitoring microbial populations
ATP bioluminescence	50-80%	Minutes	High	Quick and sensitive detection of ATP a marker for microbial activity. Useful for monitoring sanitation or detecting microorganisms in food or water samples
ELISA immunoassay	>90	Hours to days	Low	Detects specific microbial antigens or antibodies. Useful for identifying pathogens or monitoring immune responses
MALDI-TOF mass spectrometry	>90%	Minutes to hours	High	Rapid and accurate identification of microbial species based on their unique protein profiles. Useful for identifying unknown or mixed cultures
RAMAN spectrometry	>90%	Minutes to hours	High	Non-destructive, label-free detection of microbial cells based on their molecular vibrational spectra. Useful for identifying and characterizing microorganisms
FT-NIRS	50-80%	Minutes to hours	Moderate	Non-destructive, rapid detection of microbial cells based on their absorption spectra. Useful for monitoring microbial populations or identifying microbial contaminants in raw materials

PCR: polymerase chain reaction; ATP: adenosine triphosphate; ELISA: enzyme-linked immunoassay ; MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight; FT-NIRS: Fourier transform near infrared spectroscopy.

Figure 1. Schematic representation of the basic principles of FT-NIR, RAMAN spectrometry and MALDI-TOF mass spectrometry. Figures were generated by the authors using Microsoft PowerPoint 2016 and Microsoft Word 2016.



Conclusion

Microbiological contamination of pharmaceutical dosage forms is a common dilemma in the field of drug manufacturing affecting not only the aesthetic appearance of the products, but also due to its effect on the therapeutic outcomes and drug delivery pattern harboring a serious economic and health issues. Hence, pharmaceutical formulations' microbial load and contamination must be evaluated by predictive assessments. Different approaches have been employed for this purpose ranging from the traditional culture-based techniques to advanced technologies such as DNA-based PCR and MALDI-TOF mass spectrometry in addition to the immune-based method such as ELISA. Of these techniques, RAMAN spectrometry, MALDI-TOF mass spectrometry, ELISA and PCR demonstrate the highest detection rate [> 90%] while culture method and ATP bioluminescence can be relied on solely as they may miss serious contaminants. However, cost and time to get the final result are critical factors influencing the choice of the appropriate method to be used. Although preservatives are widely introduced in the pharmaceutical formulations processing to control microbial contamination the reported microbial resistance emphasizes the critical need to consider the risk of compromising formulation stability and potential health problems for consumers.

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