Specified and objectionable microorganisms

8

8.1 Introduction

This chapter focuses on the presence of specific microorganisms in active pharmaceutical ingredients, pharmaceutical products, or raw materials that might, under certain conditions, be classed as "objectionable." In many ways, the chapter is a companion chapter to Chapter 7 on bioburden determination. With specific and objectionable microorganisms, the screening and examination for such organisms normally goes hand-in-hand with bioburden testing. This is because tests for nonsterile pharmaceutical products and ingredients (such as raw materials) involve assessment of total counts and presence/absence of particular organisms of concern.

Specific microorganisms (specified microorganisms) are described in the internationally harmonized pharmacopeia (Ph. Eur. 2.6.13 and USP <62>; which harmonized in 2006) [1]. These organisms and their significance are discussed in the chapter; however, the chapter does not seek to simply regurgitate the pharmacopeia, and the reader is referred to current compendia for the test method. With the case of objectionable microorganisms, although the pharmacopeia define certain "index" or "indicator" microorganisms, contemporary approaches to risk assessment require the microbiologist to define a wider list of organisms of concern (indeed USP <1111> drew this concerns to attention in 2006) [2]. This list cannot be defined as a general selection of microorganisms for inclusion relates to specific types of products and the intended patient population for those products [3].

Thus, the concept of objectionable microorganism consists of an array that are divided between specific indicator microorganisms required for qualitative testing by the pharmacopeia and those defined as objectionable by the pharmaceutical organization in relation to a particular product.

8.2 Indicator microorganisms

Chapter 7 describes the examination of nonsterile pharmaceutical products and constituent ingredients microbial numbers (bioburden). For certain materials, there is a compendial requirement for the absence of certain microorganisms [4]. These specified microorganisms include pathogens, such as *Salmonella*, and indicators of fecal contamination, such as *Escherichia coli*. These microorganisms are specifically listed because they directly, or they may indicate the presence of other microorganism from similar sources that pose a particular risk to immunocompromised patients [5]. This is because small numbers of opportunistic pathogens become infectious when the

Pharmaceutical Microbiology. http://dx.doi.org/10.1016/B978-0-08-100022-9.00008-6

body's resistance mechanisms become impaired, through disease or as a consequence of courses of immunosuppressant drugs [6]. Indeed the risk is such that, as modeling has demonstrated, it is impossible to rule out the possibility that single pathogenic microorganism, when ingested, has the potential of inducing infection and disease [7].

These specified microorganisms are intended to be indicators of wider contamination of a type that poses a risk to human health. In essence, this means that although particular microorganisms are *specified*, there could be other microorganisms of concern that may be found in similar niches to those listed. Therefore, while it could be possible to risk assess the mere presence of a specified microorganism should it only be recovered from the sample in low numbers, the mere presence of the organism could be indicative of other microorganisms of concern to human health.

The full list of specified microorganisms described in the harmonized pharmacopeia (USP <62> and Ph. Eur. 2.6.13) is:

- bile-tolerant Gram-negative bacteria;
- *E. coli*;
- *Salmonella*;
- *Pseudomonas aeruginosa*;
- *Staphylococcus aureus*;
- *Clostridia*;
- *Candida albicans*.

These species or types of microorganisms represent:

(a) Bile-tolerant Gram-negative bacteria

With this category, the pharmacopeia have chosen a diverse grouping, and one ill-defined since there is no strict definition of this group of microorganisms. Bile-tolerant Gram-negative bacteria are best defined as those microorganisms that show growth in the stated conditions on violet red bile glucose agar medium (thus the definition is, somewhat anachronistically, centered on a culture medium). They include those Gram-negative bacteria that grow in the presence of bile salts, which are nonlactose fermenting but at the same time able to utilize glucose. Examples of some bile tolerant Gram-negative bacteria includes members of the Enterobacteriaceae and of the genus *Pseudomonads* and *Aeromonas*. In keeping with imprecise definition, there is no clear consensus as to what defines "Enterobacteriaceae" [8]. The old-fashioned categorization was of "enteric bacteria," and later of gammaproteobacteria. Conventionally this grouping includes pathogens, such as *Salmonella*, *E. coli*, *Yersinia pestis*, *Klebsiella*, and *Shigella*. Other disease-causing bacteria in this family include *Proteus*, *Enterobacter*, *Serratia*, and *Citrobacter*.

With the pharmacopeia described test, not less than 1 g of the product is enriched with an Enterobacteria enrichment broth mossel, and after incubation at 30–35 °C for a defined time, a sub-culture is performed onto violet red bile glucose agar medium.

(b) *E. coli*

E. coli is a Gram-negative, facultatively anaerobic, rod-shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts and are occasionally responsible for

product recalls due to food contamination (and on very rare occasions, pharmaceutical products) [9].

With the pharmacopeia test, both MacConkey broth and MacConkey agar are used to examine for the presence of *E. coli*.

(c) *Salmonella*

Salmonella is a genus of rod-shaped, Gram-negative bacteria. There are only two species of *Salmonella*, *Salmonella bongori*, and *Salmonella enterica*, of which there are around six subspecies and innumerable serovars. They can be divided into two groups—typhoidal and nontyphoidal Salmonella serovars. Nontyphoidal serovars are more common and usually cause self-limiting gastrointestinal disease. Typhoidal serovars include *Salmonella typhi* and *Salmonella* Paratyphi A, which are adapted to humans and do not occur in other animals [10].

The pharmacopeia test for *Salmonella* involves the use of Rappaport Vassiliadis Salmonella enrichment broth and xylose lysine deoxycholate agar.

(d) *P. aeruginosa*

P. aeruginosa is a Gram-negative, aerobic, coccobacillus bacterium with unipolar motility. It is an opportunistic human pathogen, often associated with contaminated water systems. *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, and causes blood infections. The organism is fairly straightforward to identify for *P. aeruginosa* that secretes a variety of pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin (red-brown) [11]. According to the pharmacopeia, the recommended agar for isolation and differentiation is cetrimide agar.

(e) *S. aureus*

S. aureus is a Gram-positive coccal bacterium that is frequently found in the human respiratory tract and on the skin. While *S. aureus* is not always pathogenic, it is a common cause of skin infections (e.g., boils), respiratory disease (e.g., sinusitis), and food poisoning. Disease-associated strains often promote infections by producing potent protein toxins [12]. With the pharmacopeia, the agar used for detection is mannitol salt agar.

(f) Clostridia

The Clostridia are a class of Firmicutes, including *Clostridium* and other similar genera. They are distinct from the genus *Bacillus* through lacking aerobic respiration. *Clostridium* are rod-shaped, Gram-positive endospore-forming bacteria. There are a number of species that can cause disease in humans, including *Clostridium botulinum*, *Clostridium difficile*, and *Clostridium tetani*. All pathogenic clostridial species produce protein exotoxins (such as botulinum and tetanus toxins) that play an important role in pathogenesis [13].

The pharmacopoeial test method deploys reinforced medium for Clostridia followed by Columbia agar.

(g) *C. albicans*

C. albicans is a diploid fungus that grows both as yeast and filamentous cells. It is a causal agent of opportunistic oral and genital infections in humans. *C. albicans* is commensal and a constituent of the normal gut flora comprising microorganisms that live in the human mouth and gastrointestinal tract. The fungus becomes a risk in the immunocompromised host [14].

With the pharmacopoeial method, both Sabouraud dextrose broth and Sabouraud dextrose agar are used for isolation.

8.2.1 Pharmacopeia methods

Under each section, the pharmacopeia states how much of the product or excipient is to be examined and how to incubate with each type of media with product in order to isolate any of the potential "specified" microorganisms within that product. These specified microorganism challenges must be validated to recover microbial growth as well. This portion of the microbial limits test is a presence/absence test. Depending on the product or excipient, one may choose to validate any number of the specified microorganisms from the pharmacopeia.

These bacterial and fungal indicators are selected as representatives of microorganisms that may cause disease in immunocompromised people or in other classes of susceptible persons. If such microorganisms were present, whether infection occurs, and the form it takes, depends on the route of administration, the dose of organisms, and the class of person.

Not all of these microorganisms require testing for; those that are required are described in individual monographs. This is in recognition that some types of nonsterile products are more prone to contamination than others. This reflects the point of origin or method of manufacture of the products. For example, one product prone to contamination is Arabic gum [15].

The pharmacopeia requires that where one or more of these microorganisms is to be examined, this is by qualitative analysis (a "presence–absence" test). For this, a portion of the sample (10 g or 10 mL) is incubated in broth for at least 24 h in order to enhance the isolation of any microorganisms present. The reason for incubating the samples for at least 24 h is due to the organisms, if they are present, being so in lower numbers than other types of microorganisms (for this reason identifying what is recovered for a bioburden test is insufficient since the microorganisms of concern may have simply failed to grow). An enrichment step and growth on selective media will enhance the isolation of pathogenic microorganisms.

8.2.2 Method qualification

Before sample testing is performed, the methods must be shown to be capable of detecting and isolating the specified microorganism of concern. This part of the procedure is called the preparatory testing. The preparatory testing involves the inoculation of different types of microorganisms into the samples to demonstrate the accuracy, efficacy, reproducibility, and sensitivity of a given method for detecting microbial contamination. With some products, a pretreatment test may be necessary depending upon the physical state of the product. Semisolid materials, for instance, need to be treated in order to form a solution or suspension.

It must also be established that the culture media for the test is suitable. This is affirmed by challenging each medium with a suitable panel. A test panel will include those microorganisms that should grow on the medium; microorganisms where

growth on the medium reveals particular indicative properties, such as certain colonial pigmentation; and microorganisms that should not be recovered on the medium because the medium is intended to be inhibitory. For example, taking mannitol salt agar, which is used for the test for *S. aureus*, then the appropriate control organism for growth promotion and indicative growth is, unsurprisingly, *S. aureus*; whereas the organism for the test for inhibition is *E. coli* (where *E. coli* should not be recovered on the agar).

8.3 Determining which microorganisms are objectionable and assessing risk

Rigidly testing for the microorganisms listed in the compendia may not be the correct strategy. This is because it is recognized that there may be other "objectionable" microorganisms that are more appropriate and pose a greater risk to the product and therefore to the patient [16]. With this regard, the harmonized pharmacopeia requires that the significance of other microorganisms recovered should be evaluated. This is also in keeping with the requirements of the FDA Code of Federal Regulations (CFR). These missives are:

- 21 CFR 211.84(d)(6)—"Each lot of a component, drug product container, or closure with potential for microbiological contamination that is objectionable in view of its intended use shall be subjected to microbiological tests before use."
- 21 CFR 211.113(a)—"Appropriate written procedures, designed to prevent objectionable microorganisms in drug products not required to be sterile, shall be established and followed."
- 21 CFR 211.165(b)—"There shall be appropriate laboratory testing, as necessary, of each batch of drug product required to be free of objectionable microorganisms."

In keeping with risk assessment methodologies, it is incumbent upon the pharmaceutical organization to define their own problematic microorganisms [17]. Assessing whether a microorganism is objectionable requires the assessment of a number of factors. The foremost factor is whether the organism is a pathogen for if the organism is known to be a pathogen, and the route of infection is the same as the route of administration for the product, the organism is most likely objectionable.

Evaluation of whether a microorganism is or is not objectionable should include the following:

- The use of the product and the method of application (eye, nose, respiratory, dermal, and so on) in relation to different microorganisms. This is because different microorganisms carry differing risks depending upon the way that the product is taken by the patient;
  - For example, with oral products: *Candida* species, aflaxtoxin-producing *Aspergillus* species, *Bacillus cereus*, *Burkholderia cepacia*, Enterobacteriaceae (such as *Klebsiella* species), and other microorganisms were the population exceeds 100 CFU;
  - Some products carry a higher risk than others, for example, inhalation products and nasal sprays, optics, vaginal and rectal products, and oral solutions;
- The nature of the product. This involves considering whether the product supports growth, and if so whether it will support certain microorganisms more than others?

- The intended recipient: risk may differ for neonates, infants, the debilitated;
  - Limits for objectionable microorganisms in oral products intended for use by immunocompromised patient populations such as pediatric, human immunodeficiency virus (HIV), and cancer must be tighter than the limits for oral products intended for treating patients with diseases or conditions not affecting their immune systems because patients with deficient immune systems are more at risk of microbial infections [6];
- The presence of disease, wounds, organ damage;
- Where warranted, a risk-based assessment of the relevant factors is conducted by qualified personnel.

The list of objectionable microorganisms generated from a review should not remain static. The use of an experienced microbiologist to regularly screen and assess the microorganisms found in product and recovered from the environment needs to form part of an organization's continual risk assessment process.

To add to the complexity, a given microorganism may become "objectionable" under certain circumstances. The ways by which objectionable microorganisms trigger a risk to the product or have potential to cause patient harm include:

1. affecting product stability;
2. affecting the security of the container/closure system (is, for instance, the container adequately designed to retard access to the environment, and to prevent contamination from the environment?);
3. affecting the active ingredient;
4. producing off odors, flavors, or undesirable metabolites;
5. having the potential to grow and exceed the total aerobic count specification;
6. possessing high virulence and a low infective dose;
7. resistance to antimicrobial therapy.

When an objectionable microorganism is detected, this may or may not lead to rejection of the affected lot. To decide whether lot rejection must occur is dependent upon risk assessment. In risk assessing the impact of a microorganism on the product or process, a number of steps are required. These are [18]:

- Identity of the microorganism: find as much information as possible about the organism. This includes looking at recalls and other industries (e.g., medical and food) to determine if it could be a pathogen.
- Number of microorganisms present in the product: it is important to know the number of organisms present, especially when considering the infective dose. In addition, an assessment of the total number is important even if the microorganisms detected are not considered to be pathogenic. High numbers of nonpathogenic organisms may affect product efficacy and/or physical and chemical stability. It also stands that an unusually high number of microorganisms seen in the product may also indicate a problem during the manufacturing process;
- Microbial toxins. With this it is necessary to consider if the microorganism is likely to release a toxin (exotoxin, enterotoxin, or endotoxin) that could cause patient harm even if the microorganism is no longer viable.
- Consider the nature of the product: does it support growth? Does it have adequate preservation? Is it aqueous, cream, suspension, etc.?
- Assess the capability of the product to support growth or sustain the microorganisms. This requires knowledge of the inherent product characteristics, such as pH, water activity, and osmotic pressure. It should be noted that with the more resistant microorganisms, including

spore-forming bacteria, although they may not proliferate in a drug product with a low water activity, may persist within the product for long periods. In terms of some of the product characteristics:

  - reduced water activity will greatly assist in the prevention of microbial proliferation in pharmaceutical products; and the formulation, manufacturing steps, and testing of nonsterile dosage forms should reflect this parameter [19];
  - with pH, it should be assessed if the product pH in the same range as the ideal growth pH for the organism in question?
  - with the product in general, it is important to assess of the product formulation contains ingredients that would be antimicrobial for the microorganism?
- With raw materials: consider processing to which the product is subjected, current testing technology and the availability of materials of desired quality.

In reviewing the outcome of the above evaluation: "is the microorganism objectionable?" and "when an objectionable microorganism is found, what is the risk?", a microorganism is likely to be classed both as objectionable and a high risk, if:

- the identification of the species has been confirmed;
- the patient population does not exclude those susceptible to the illness that this organism causes;
- the microorganism is known to cause illness;
- product route of administration is the same as the organism's route of infection (e.g., the bacterium causes illness via ingestion and the product is an oral product);
- the infective dose is low;
- it takes only a few cells to cause illness;
- it cannot be proven that the organism will not proliferate in the product.

With the above criteria, should they be met in whole or in part, then there would be little choice other than to reject the product.

8.4 Human microbiome project

With the establishment of the human microbiome project (HMP), knowledge of the diverse span of microbial species within and across the human body has been significantly enhanced, revealing valuable insight into community niche specialization, genetic diversity, and the prevalence of indigenous opportunistic pathogens. The HMP began in 2008 as a US National Institutes of Health initiative. The core objective of is to identify and characterize microorganisms associated with both healthy and diseased humans (the human microbiome) using a combination of culture techniques, metagenomics, and whole genome sequencing.

Arguably the outcomes from the analysis of the HMP have expanded the types of microorganisms that are considered to be objectionable. For example, upon exposure to pharmaceutical therapies containing antimicrobial preservatives, the diversity and composition of the human microbiome can be compromised, potentially resulting in physiological changes or the overgrowth of opportunistic pathogens. Conversely, the microbiome itself can also influence the human physiological response to pharmaceutical products, thus affecting the intended function of the product. A third consideration

is that members of the human microbiome could be shed or deposited during the manufacturing process, thereby becoming an inadvertent source of contamination [20]. Thus, keeping abreast of the developments in this field is required in order for risk assessments in relation to objectionable microorganisms to be meaningful.

8.5 Conclusion

This chapter has presented a discussion about particular microorganisms and pharmaceutical products. This has centered on those microorganisms that are specified in the pharmacopeia as indicators of contamination and those that each facility must separately consider as "objectionable." Sometimes the self-assessed objectionables are the same as the compendial species and, at other times, they will be different.

The chapter has also presented approaches that can be taken for considering which organisms could be classed as objectionable and then, should such organisms be detected in a sample, how the impact of the detection can be risk assessed in terms of whether the material from which the sample was taken should be rejected.

This represents an important area since regulatory citations are relatively common in relation to objectionable microorganisms. Citations often center on the characterization of an objectionable microorganism in view of the product's intended use, the patient population (such as age and gender), patient health, dose, and application frequency of the medicine. Thus, the risk assessment process to enable assessments to be made needs to be scientifically sound and up-to-date.

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