Figure 1: Typical settle and contact plates

A strategy for microbe monitoring

Key to any cleanroom or aseptic operation is a planned programme of monitoring. Colin Booth, vice-president science and technology at Oxoid, outlines the basis for a microbial monitoring strategy

Where it is not possible to terminally sterilise, to be confident of producing a sterile product manufacturers of parenteral drugs must ensure that a robust environmental monitoring programme is in place within aseptic processing areas (cleanrooms).

According to the US Food & Drug Administration, environmental monitoring should promptly identify potential routes of contamination, allowing for implementation of corrections before product contamination occurs.[1] It is one of the most important laboratory controls in aseptic processing, providing crucial information on the quality of the aseptic manufacturing environment; preventing the release of a potentially contaminated product; and preventing future contamination by detecting adverse trends.

Ideally, a monitoring programme provides immediate feedback, allowing any problem to be rectified promptly. Environmental monitoring by microbiological means does not provide this because of the incubation time required to detect micro-organisms. Nevertheless, the microbiological status of the cleanroom is a valuable tool that can indicate a loss of environmental control not picked up by other means. It also serves as a reminder to personnel of the importance of aseptic techniques.

Environmental monitoring is only a part of ensuring a sterile manufacturing environment. Other elements include: good cleanroom design; effective air handling; cleaning and disinfection; staff training for adherence to aseptic techniques; properly fitted cleanroom gowns; sterilisation of materials brought into the aseptic environment; adequate monitoring of controlled conditions (such as air flow and air pressure) along with alert settings that draw attention to a developing problem.

There are many routes by which a cleanroom can become contaminated, personnel being one. In a well-designed and controlled cleanroom, personnel are the most likely source of contamination. This may originate in the changing room where personnel shed their own clothes and put on sterile suits. If this process is not performed correctly, contaminants can enter the cleanroom on gowns, gloves, overshoes, etc. The garments worn in the cleanroom must fit well, so that skin particles are retained within the suit, and be made of a material that does not shed fibres or particles. Aseptic techniques should be taught in order that personnel do not contaminate gloves after, for example, touching their faces.

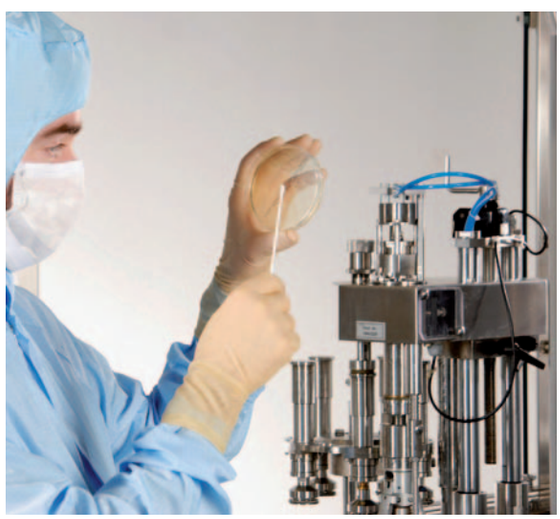

Figure 2: Swabbing is used to monitor areas that are difficult to access

Water is a potential vector of contamination, enabling organisms to spread in ways they cannot on solid surfaces. Regulations state that there should be no water outlets in cleanroom areas; however, water is used in cleaning and is probably the most commonly used raw material in manufacture. Water-based fluids must be sterilised before they are taken into the filling rooms. As water is also used in cleanroom support areas, pharmaceutical grade water should be used wherever possible, and it should be microbiologically controlled and monitored.

Materials of animal, plant or natural mineral origin are considered to be of higher risk than materials produced by chemical synthesis. However, any materials introduced to an aseptic processing area should be monitored for signs of damage and attention paid to the microbiological quality of the material content.

Disruption of airflow, air movement and inadequate maintenance of HEPA filters could all cause contamination. HEPA filters should be free from leaks around the sealing gaskets, frames and fabric. A high degree of air movement can pick up skin or dust particles from personnel or equipment, so cleanroom design should ensure that airflow sweeps particles away from the point-of-fill, product or other critical areas. Proper design will ensure uni-directional airflow with no turbulence or stagnant air.

Although it is well known that the design and construction of the cleanroom should involve smooth surfaces made of easy-to-clean materials, special attention must be given to areas that are difficult to clean and disinfect. Different control measures should ▶

be in place to minimise contamination from these sources.

The aim of an environmental monitoring programme is to detect a change in count of colony forming units (cfu), or a change of species, outside of the 'norm'. Minimum acceptable standards for clean areas are laid down in guidance, such as the US Pharmacopoeia (USP), the EU (2003) guide to Good Manufacturing Practice, Annex 1, Manufacture of Sterile Medicinal Products, (see table 1) and the Manufacture of Sterile Products 2002 (Therapeutic Goods Agency, Australia).

The inspecting body will determine the guidance that should be followed for each site. Each site should establish its own alert and action levels based on the historical data. These must be at least as strict as in the standard. If the data suggests that local alert and action levels should be stricter, a regulatory body would expect these stricter levels to be adopted.

The most common micro-organisms isolated from aseptic processing environments are bacteria from human skin, such as Staphylococcus and Micrococcus species (gram-positive cocci). In addition, airborne bacterial spores (e.g. Bacillus species) and fungal spores (e.g. Aspergillus or Penicillium species) are found and, more infrequently, gram-negative cocci (e.g. Enterobacter and Burkholderia species).[2]

The data produced, both counts and species, leads to a greater overall understanding of the microbial environment within the manufacturing area and, if performed regularly, can reveal trends or changes in established patterns. This information can be used to identify high-risk areas and to eliminate potential routes of contamination.

There are two main methods for sampling air for microbial contamination: active and passive. Active air sampling is generally considered to be quantitative as it measures the number of viable organisms in a defined volume of air (e.g. cfu/m^3). Several types of active air samplers are available, including sieve impactors, slit-to-agar samplers, surface vacuum samplers, centrifugal impactors, filtration or liquid impingers and hand-held samplers.

In a slit-to-agar sampler an agar plate (usually 140mm) is placed on a turntable and spun underneath a template with four perpendicular slits in it. Air is drawn in through the slits by means of a pump attached to the unit by a flexible hose. The air impacts on the surface of the agar plate, as do any airborne particles. Integral, hand-held units, contain both in the body of the device and pump. The agar plate sits in the head of the machine, which has a perforated cover through which the air is drawn. The air impacts on the surface of the agar, along with the airborne particles, before being expelled through the rear of the machine.

Passive air sampling is performed by placing agar plates (see Figure 1) in test areas, with the agar surface exposed for a set time. In 1981, the Parenteral Drug Association (PDA) recommended only 30 minutes, but more recently other guidelines recommended that this be increased to four hours. The logic is that longer exposure times increase the sensitivity of the method. However, care has to be taken against excessive desiccation of the medium, since this may reduce recovery rates.

Although passive air sampling is not quantitative, settle plates are relatively inexpensive, require no extra equipment or technical expertise and are less likely to disturb the airflow in the cleanroom than active air sampling.

With either method, the frequency of testing should take into account the fact that any human intervention introduces an increased risk within critical areas. The manufacturer must weigh the benefits of these interventions against the disruptions created by performing them.

surface samples

Regular sampling of surfaces within the processing environment is another important aspect of monitoring an aseptic environment. However, the monitoring of critical surfaces during production is not recommended as this can increase the risk of contamination. Surfaces can be sampled using contact plates (Figure 1) or swabs (Figure 2).

Contact plates are used to sample flat surfaces on equipment, floors, walls, countertops, etc. These specially designed agar plates have a domed surface that is pressed gently onto the surface causing organisms present to be transferred to the agar. This method is mentioned within the pharmacopoeias, and is considered quantitative as it measures the number of viable organisms on a defined surface area.

Surfaces that are not flat or are difficult to access may be sampled using swabs. It is important to sample such areas as they may be more difficult to clean. The surface area to be tested is sampled with a moistened swab. This can be used to inoculate a plate directly or it can be immersed in a diluent or transport medium, which is then used to inoculate a plate. Swabs are not mentioned in the pharmacopoeias but they are effective for sampling areas that are not suitable for contact plates. For either method, neutralisers may be included in the medium to counteract residual sterilants and cleaning agents that may be present on test surfaces.

The effectiveness of aseptic training and precautions can be measured by periodically sampling staff clothing. The hands are a potential source of contamination and gloves should be tested periodically – this is done by touching the tips of all fingers and thumbs onto the surface of an agar plate, after which the gloves are replaced with a clean set. At the end of a shift, other garments can be tested using contact plates or swabs.

The most frequently used culture media in environmental monitoring are Tryptone Soya Agar (TSA) – a general purpose medium suitable for the growth of a wide range of organisms, and Sabouraud Dextrose Agar (SDA) – ideal for the growth of yeasts and moulds. Other key media used include: R2A Agar (a minimal medium for testing water samples); McConkey

Table 1: Recommended limits for microbial contamination in clean areas levels (EU (2003) guide to GMP, Annex 1)

Classification of clean area	Active air (cfu/m^3)	Settle plate (90mm) (cfu/4hr exposure)	Contact plate (55mm) (cfu/plate)	Glove print (cfu/glove)
Grade A (including local zones for high risk operations, eg, point of fill)	<1	<1	<1	<1
Grade B (background environments to Grade A zones in a cleanroom)	10	5	5	5
Grade C (support areas, eg, rooms where aseptic solutions are prepared for filtration)	100	50	25	N/A

Validating media performance

The growth promotion properties of each medium used should be validated, according to plate size and fill volume, using a pre-defined list of organisms. This list should include compendial organisms and environmental isolates and should be representative of those found in manufacturing environments, including gram-positive rods, gram-positive cocci, filamentous fungi and gram-negative rods.

The laboratory must have a robust method for the storage and recovery of organisms. For standard strains, the use of convenient, commercially available organisms, such as Quanti-Cult (Oxoid), is recommended.

Once an agar plate has been inoculated with 10-100 cfu of the test organism, it should be incubated at an appropriate temperature for up to 48 hrs for bacteria or up to 3-5 days for fungi. The recovery rate for unstressed organisms on general media should be at least 70%, and for selective media it should be at least 50%.

Agar (coliforms); XLD Agar (salmonellae); Cetrimide Agar (*Pseudomonas aeruginosa*); Mannitol Salt Agar (staphylococci); DCA Agar (intestinal pathogens); Baird-Parker Medium (*Staphylococcus aureus*).

Ready-to-use prepared media products are now popular since they provide consistent product performance. Pharmaceutical companies are expected to QC the media that they use, using quantitative methodology and low level inocula. It is, therefore, advantageous if prepared media has been tested in this way and is supplied with supporting documentation.

Prepared plate media are normally supplied in 90mm or 140mm Petri dishes or contact plates. A variety of irradiated products are available, suitable for use in cleanroom areas. Impermeable isolator wrap is available that allows plates for environmental monitoring to be left in an isolator unit while it is being flushed with sterilants, such as vapour-phase hydrogen peroxide.

programme design

In designing an effective environmental monitoring programme one should consider:

1. A strategy – a written action plan, including where and when to test, focusing on known weak areas in addition to searching for new ones.

2. Validation – all methods used in the programme should be validated, including: media stability; the maximum autoclave cycle; media performance (see Figure 3); effectiveness of neutralisers (to demonstrate that the disinfectant, at the concentration used in the facility, is inactivated). It may also be necessary to note the expected morphology of certain organisms, since the morphology of colonies grown on irradiated agar plates may be different from those grown on fresh agar.

3. Standard operating procedures – including the purpose of the task; frequency of assay; composition of media; selection of control strains; incubation; criteria for release; documentation; action on 'out of spec' results.

4. Growing/preparing isolates – environmental isolates will often be stressed, therefore subculture is required to allow the organisms to recover, to obtain a pure culture and to produce enough material to perform an identification.

5. Identification – all isolates from Grade A environments should be identified to species level; morphologically representative isolates from Grade B areas should be identified to species level; morphologically representative isolates from Grade C & D areas should be characterised if above alert levels.

Periodic characterisation should be performed if levels fall outside alert levels. Identification can be performed using biochemical or serological methods, although genetic techniques are being used more frequently as they are not affected by the stress status of the test organism. Sub-species characterisation is possible using the Riboprinter System (Oxoid), an automated riboprinting (DNA sequencing) method.

With all elements of a good environmental monitoring programme in place, manufacturers have assurance that control of the aseptic process is being maintained. Counts that fall outside acceptable levels do not necessarily mean that product will be contaminated, but it allows the process to be examined to identify areas where control is lost. Adverse trends can be identified quickly, allowing potential future problems to be averted. Failure to do this can have serious consequences – from an FDA 483 report to possible product recalls. ■

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