NHS PQAC Advice Note

Incubation Regimes for Microbiological Media

Introduction

Hospital cleanroom environments employ a variety of methods of microbiological environmental monitoring (finger dabs, settle plates, contact plates, swabs and active air samples) to ensure compliance with regulations (e.g. EU cGMP limits[1]) and to be able to see that the environment, facilities and personnel remains in control. The results of the monitoring methods are used to detect trends in the contaminants recovered to ensure that early action is taken.

The growth media chosen for an environmental monitoring program depends on the time-temperature incubation regime to be employed. To reduce the number of samples used, and the number of invasive procedures in an aseptic clean room, many sites choose to use a general purpose media e.g. Tryptone Soy Agar (TSA) and employ a **dual temperature incubation** regime which allows the recovery of aerobic bacteria and fungi (yeasts and moulds).

Dual temperature incubation regimes employ a fixed or minimum period at one set temperature followed by a fixed or minimum period at a higher or lower temperature. A number of incubation regimes have been developed by NHS QC Laboratories, based on knowledge of the environment being monitored and local research.

Most dual temperature regimes employ a period at 20-25°C and a period of 30-35°C, either:

- Low-High Incubation e.g. 5 days at 20-25°C followed by 2 days at 30-35°C
- High-Low Incubation e.g. 2 days at 30-35°C followed by 5 days at 20-25°C

The incubation time and temperature can also vary; for example some laboratories have equal time allocated to each temperature range e.g. 3 days at 20-25°C followed by 3 days at 30-35°C.

The sequence of these two regimes has been questioned by the MHRA, who prefer the Low-High incubation regime to the High-Low regime, but there appears to be very little official advice or definitive evidence relating to the order of incubation or the time period at each temperature range.

To answer this the National Microbiological Protocols Group attempted to determine if there is any statistical difference between these regimes and to establish if there are any good reasons to employ both, on occasions, depending on the environmental results already established.

Evidence

A recent paper by Dr. Tim Sandle[2] compared the two following incubation regimes using TSA:

- Incubation for a minimum of 2 days at 30-35°C, followed by a minimum of 5 days at 20-25°C (H-L)
- Incubation for a minimum of 5 days at 20-25°C , followed by a minimum of 2 days at 30-35°C (L-H)

The study showed that there was **no significant difference** between the total colony count results (bacteria and fungi) of the two incubation regimes. Although not significantly different, the second regime (L-H) gave higher mean counts than the first regime (H-L).

When **fungal** colonies alone were considered, a higher number of colonies were recovered from the second regime (L-H) and this was proven to be statistically significant.

These findings compare well results reported by Gordon *et al*[3] and similar work carried out at the Nottingham University Hospitals QC Laboratory (NUH)[4] which considered the following two regimes:

- Incubation for a minimum of 3 days at 30-35°C, followed by a minimum of 3 days at 20-25°C (H-L)
- Incubation for a minimum of 3 days at 20-25°C, followed by a minimum of 3 days at 30-35°C (L-H)

In this case there was **no significant difference** to the number of **bacterial** colonies recovered (Student's t-test). Although this study used TSA plates exposed in uncontrolled areas in an attempt to increase the total numbers of both bacterial and fungal colonies recovered, in practice quite small numbers of fungal colonies were recovered. Because the number of fungal colonies obtained was low, statistical analysis could not be undertaken. However, larger numbers of fungal colonies were recovered from the Low-High regime, where lower temperature incubation was carried out first.

Other work carried out by a group in the USA[5] which focussed on the recovery of fungal organisms showed that the selection of suitable media was as important as the choice of incubation temperature. This group also showed that a wide range of fungal contaminants found in clean room environments could be reliably recovered on TSA supplemented with Lecithin and Polysorbate 80 incubated at elevated temperature for a shortened period i.e. 70-72 hours at 30-35°C. This compared well to 4-5 days at 20-25°C on Sabouraud Dextrose Agar (SDA).

Advice

The studies by Sandle[1]; Gordon *et al*[3] and NUH[4] would appear to endorse the MHRA preference.

If dual temperature incubation regimes are used, the lower temperature should be employed first.

This is particularly useful if no other selective media is used for the recovery of fungal contaminants e.g. SDA.

If the environment monitoring results suggest that fungal colonies are present, the Low- High incubation regime would be preferable.

If fungal colonies are rarely encountered and a bacterial monitoring result is required quickly, then the High-Low incubation regime can be recommended.

The periodic monitoring with a selective media for fungi should be used also; so that any increase in fungal contamination can be detected and acted upon.

NHS PQAC Advice Note
Date of Issue: 25th November 2014.

NHS Pharmaceutical Micro Protocols Group
Bernie Sanders & Tim Sizer; John C Rhodes; Sarah Hiom; Linda Musker; Janet Shaw; Mark Oldcorne; Ayo Ogunsanlu

Supplementary Advice:

With either dual incubation regime there are advantages and disadvantages to be considered:

Regime	Advantages	Disadvantages
H-L High-Low	Fungal colony size restricted [5] (prevents overgrowth on plate)	Lower number of fungal colonies recovered.[2,4]
	Bacterial contaminant numbers available sooner enabling interim reporting.	
L-H Low- High	Higher number of fungal colonies recovered.[2,4]	Fungal colony size may restrict visibility of other fungal or bacterial colonies.
		Bacterial results not available until the end of both incubation periods.

Handling and Transport of Microbiological Media

Whichever incubation regime is employed, there are a number of points which should be considered when storing, transporting and incubating microbiological media to ensure full and reliable results are obtained:

- Before use, plates should be stored according to the manufacturer's instructions. Refrigeration is not normally recommended as this encourages condensation under the lids
- Agar plates should be unwrapped in a controlled environment (Grade B or Grade A). Unpacking and labelling should be avoided until needed for use and this should not be undertaken in 'busy' areas
- During transportation to the aseptic unit plates should be protected from light, excessive temperatures and external contamination.
- During incubation plates which are wrapped too tightly (hermetically sealed) may engender excessive condensation, which can cause random multiplication of colony forming units (cfus) when changing temperature. Use of plastic bags, Sellotape®, parcel tape and similar are particularly prone to this problem
- Where plastic bags are used, these should be either new or sterile.
- Elastic bands to hold plates together are acceptable, but an overwrap is desirable, to prevent contamination from external sources during transit. Cling film has been found to be useful
- Incubation at 20-25°C should be for a minimum of 5 days
- Movement of plates during incubation should be limited and carried out carefully to minimise spreading of colonies by spores or droplets, resulting in increased counts. This is important when transferring plates from one incubator to another and/or carrying out an interim read.
- Plates should be incubated in the inverted position, to reduce the risk of condensation falling onto the agar surface.

Please Note: The micro Protocol Group is continuing investigations into whether there are benefits for two temperature incubation over a single incubation at 25°C and comparing incubation time periods. This will be communicated in due course.

References

1. (EU GMP) Rules and Guidance for Pharmaceutical Manufacturers and Distributors 2014: See Annex 1 sections 19 and 20.
2. Sandle T; Examination of the order of incubation for the recovery of bacteria and fungi from Pharmaceutical-grade cleanrooms. *Int. Journal of Pharmaceutical Compounding Vol.18 No.3 May/June 2014.*
3. Gordon O, Berchtold M, Steark A & Roesti D; Comparison of different incubation conditions for microbiological environmental monitoring *PDA J Pharm Sci and Technol. 2014, 68(5) 394-406*
4. Environmental Monitoring Incubation Sequence Investigation; September 2014. QC Unit, Nottingham University Hospitals NHS Trust. Contact: Sanders B.
5. Marshall V, Poulson-Cook S, and Moldenhauer J; Comparative mold and yeast recovery analysis (The effects of differing incubation temperature ranges and growth media)
   PDA J Pharm Sci and Technol. 1998, 52(4) 165-169