PII-10

Use of HEPA Filtration within an Anaerobic Chamber to Reduce Bacterial Density in the Incubation Atmosphere

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Abstract

The presence of bacterial cells or spores within the atmosphere of an anaerobic chamber may cause cross contamination between cultures, present a risk to the operator and lead to ejection of viable bacteria into the external environment via excess gas or water released from the chamber.

We have developed a prototype anaerobic workstation with an integral, custom-made HEPA filter that achieves 0.3 µm particle counts better than ISO 14644-1 Class 3 within the chamber.

We evaluated the removal of vegetative bacteria (*Kocuria rhizophila*) and spores (*Clostridium beijerinckii*) from the incubation atmosphere of this workstation during normal operation and compared its performance with an equivalent model not equipped with a HEPA filter.

Before testing, each workstation was allowed to stabilize at 37°C and 75% relative humidity and was operated continuously throughout the test so that normal atmospheric circulation occurred. Suspensions of *K. rhizophila* cells and *C. beijerinckii* spores were aerosolized using a 6 jet Collison nebulizer within each workstation over a 5 minute period. This introduced viable counts of >10^8 cfu (*K. rhizophila* cells) or >10^5 cfu (*C. beijerinckii* spores) into the chamber.

Viable bacteria in the incubation atmospheres were subsequently enumerated using an air sampler and appropriate agar plates. Viable bacteria expelled through the pressure relief valves were also collected using an air sampler, while condensate water (pumped out of the workstations during normal use) was spread onto agar plates for bacterial enumeration. No viable bacteria were recovered from the HEPA filtered workstation 5 minutes after aerosolization, while bacteria remained abundant in the standard workstation for at least 4 hours. In addition, viable bacteria were recovered from the pressure relief valve of the standard workstation but not from the HEPA filtered unit. Thus, the use of HEPA filtration in an anaerobic chamber produces a substantial reduction in bacterial contamination of the atmosphere and could potentially reduce ejection of bacteria into the surrounding laboratory environment.

Materials and Methods

The standard anaerobic chamber (without HEPA filtration) used in our experiments was the Whitley A35 Anaerobic Workstation (Don Whitley Scientific Limited, Shipley, UK). The prototype HEPA filtered chamber is based on the A35 workstation, with an enlarged acrylic shell to accommodate a bespoke HEPA filter, which is fitted within the internal atmospheric flow path. The standard circulation system was redesigned to compensate for the flow restriction produced by the HEPA filter. Continuous recirculation of the chamber atmosphere through the filter ensures that particulate matter is captured within the filter and prevented from further movement. The entire internal atmospheric volume passes through the filter every 4 seconds.

Prior to bacteriological testing, particle counts in the HEPA filtered workstation (at rest) were determined using a CI-154 particle counter (Climet Instruments Co., Redlands, CA). On the basis of particle counts in the range 0.3 µm to 5.0 µm, the chamber attained levels of atmospheric cleanliness exceeding ISO 14644-1 Class 3 (Class 1 of US Fed. Std. 209E).

Biological testing of the two chambers was performed using bacterial strains with low pathogenic potential (Risk Group 1). A non sporeforming aerobic species (*Kocuria rhizophila*) and a sporeforming anaerobe (*Clostridium beijerinckii*) were used. To produce a suspension of vegetative cells for aerosolization, *K. rhizophila* was subcultured on Tryptone Soy Agar (TSA) at 37°C for 2 days. Cells were harvested into sterile 0.85% NaCl using a "hockey stick" plate spreader. To produce a spore suspension, *C. beijerinckii* was cultured anaerobically in 2 × 500 ml volumes of Bryant and Burkey medium at 37°C for 10 days. The two liquid cultures were heated at 80°C for 10 minutes, centrifuged to pellet the spores and cell debris, washed 3 times in sterile 0.85% NaCl and the combined material from both cultures was resuspended in 100 ml of sterile 0.85% NaCl to concentrate the spores. Viable counts (determined by plating) were >1×10^8 cfu/ml for *K. rhizophila* cell suspension and >1×10^5 cfu/ml for *C. beijerinckii* spore suspension.

Before testing, each anaerobe chamber was allowed to stabilize at 37°C and 75% relative humidity and was operated continuously throughout the test so that normal atmospheric circulation occurred. A 55 ml volume of *K. rhizophila* cell suspension or *C. beijerinckii* spore suspension was added to a 6 jet NSF Collison nebulizer (BGI Inc., Waltham, MA) and the fully assembled nebulizer was weighed. The nebulizer was transferred into the chamber, placed on the upper shelf with the outlet facing the front of the chamber and connected to compressed air at 20 psi/12 litres per minute. Nebulization was performed for 5 minutes, after which the nebulizer was weighed again to determine the dispensed volume.

Atmospheric sampling was conducted using an AES Sampl'air Lite (bioMérieux, Combourg, France). Samples of 100 litres were collected by impaction onto a 90 mm petri dish of TSA (*K. rhizophila* experiments) or Fastidious Anaerobe Agar (FAA; *C. beijerinckii* experiments). Samples were collected during the fourth minute of nebulization, immediately after completion of nebulization and at timed intervals during the subsequent 30 minutes, at the following locations in both workstations:

- **Chamber interior** - shown in red in the tables of results
- **Adjacent to the pressure relief valve outlet on the chamber exterior** - shown in blue in the tables of results
- **External atmosphere adjacent to the chamber, in the operator's position** - shown in purple in the tables of results.

The outlet of the condensate pump on each workstation was sampled by switching the pump on manually and holding an appropriate agar plate under the condensate outlet for 1 minute.

Exposed plates from the air sampler and condensate pump were incubated for 2 days at 37°C aerobically (*K. rhizophila* experiments) or anaerobically (*C. beijerinckii* experiments) and colonies were enumerated.

Results and Discussion

Recovery of bacterial colonies from the chamber interiors, pressure relief valves and external environment is presented in the tables.

Sampling of the chamber's internal atmosphere demonstrated that the standard anaerobic workstation allowed *K. rhizophila* to persist (without a measurable reduction in viable count) for at least 30 minutes after nebulization ceased. An additional sample after 4 hours (data not shown) also revealed high numbers of the organism. In contrast, *K. rhizophila* count in the atmosphere of the HEPA filtered chamber 2 minutes after nebulization was reduced more than 100-fold in comparison with the standard chamber and the organism could not be recovered after 5 minutes. This exceeds the requirement, stipulated in ISO 14644, that the appropriate class of atmospheric cleanliness should be regained within 20 minutes following any disruption.

Sampling of the pressure relief valve outlet and the external atmosphere in the operator's normal working position demonstrated that *K. rhizophila* was ejected in large numbers from the standard anaerobic chamber. Sampling of these positions outside the HEPA filtered chamber recovered no bacterial colonies during the nebulization process and at all sample points up to 30 minutes.

To test the capacity of the HEPA filter to remove bacterial spores, the nebulization experiment was repeated using a suspension of *C. beijerinckii* spores. Sampling of the chamber's internal atmosphere after nebulization of this suspension produced a small colony count immediately after the nebulizer was stopped, then no recovery of the organism after 2 minutes. Thus, the HEPA filter was also highly effective in the removal of spores from the atmosphere (the shorter time required to achieve zero counts, in comparison with the results obtained for *K. rhizophila*, was probably attributable to the lower viable count attained in the spore suspension).

Following nebulization of bacterial cells or spores, no colonies were recovered from the condensate outlets of either workstation (data not shown in tables). Thus, this does not appear to be a route for egress of viable bacteria from the chamber.

Thus, our experiments have demonstrated that the use of HEPA filtration in an anaerobic chamber produces a rapid and substantial reduction in bacterial contamination of the atmosphere. A HEPA filtered anaerobic chamber could potentially be useful in several areas of microbiology:

(i) Use of anaerobic culture as part of a pharmaceutical manufacturing process, which would ideally be conducted under "cleanroom conditions". Example would include the growth or manipulation of anaerobes used to produce a vaccine or pharmaceutical preparation.

(ii) Cultivation of slow-growing anaerobes: HEPA filtration would reduce the risk of contamination by faster growing strains.

(iii) Application requiring complex manipulations under anaerobic conditions (for example, biochemical assays) where anaerobe cultures may be open to the ambient atmosphere for an extended period. HEPA filtration would reduce the risk of contamination under these conditions.

Although atmospheric cleanliness (removal of particulate matter and viable organisms) was the primary objective in our development of a HEPA filtered anaerobe chamber, the configuration used for our experiments also demonstrated reduced ejection of bacteria into the surrounding laboratory environment. This could have an obvious biosafety role in applications with hazardous organisms or when aerosols are likely to be formed.

Additional product development projects are planned to further enhance atmospheric filtration features.

Results

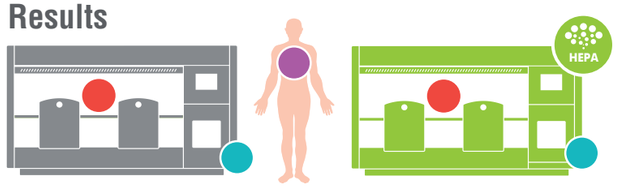

Table 1: Recovery of *K. rhizophila* colonies from standard anaerobic chamber (No HEPA)

Before nebulization	During nebulization (1.9x10^8 cfu / 5 min)	After nebulization						
		0	2 min	5 min	10 min	15 min	20 min	30 min
0	≥258	≥258	≥258	≥258	≥258	≥258	≥258	≥258
0	≥258	≥258	≥258	≥258	≥258	≥258	≥258	≥258
Before nebulization	During nebulization (1.2x10^8 cfu / 5 min)	0	2 min	5 min	10 min	15 min	20 min	30 min
		After nebulization						

Table 2: Recovery of *K. rhizophila* colonies from HEPA filtered anaerobic chamber

Before nebulization	During nebulization (2.1x10^8 cfu / 5 min)	After nebulization						
		0	2 min	5 min	10 min	15 min	20 min	30 min
0	≥258	55	2	0	0	0	0	0
0	0	0	0	0	0	0	0	0
Before nebulization	During nebulization (1.2x10^8 cfu / 5 min)	0	2 min	5 min	10 min	15 min	20 min	30 min
		After nebulization						

Table 3: Recovery of *C. beijerinckii* colonies from HEPA filtered anaerobic chamber

Before nebulization	During nebulization (3.3x10^5 cfu / 5 min)	After nebulization						
		0	2 min	5 min	10 min	15 min	20 min	30 min
0	232	8	0	0	0	0	0	0

Table 4: Recovery of *K. rhizophila* colonies from operator's position (No HEPA)

Before nebulization	During nebulization (1.9x10^8 cfu / 5 min)	After nebulization						
		0	2 min	5 min	10 min	15 min	20 min	30 min
0	≥258	≥258	≥258	≥258	≥258	≥258	≥258	≥258

Table 5: Recovery of *K. rhizophila* colonies from operator's position (HEPA)

Before nebulization	During nebulization (1.2x10^8 cfu / 5 min)	After nebulization						
		0	2 min	5 min	10 min	15 min	20 min	30 min
0	0	0	0	0	0	0	0	0

Numbers of colonies recovered are all from 100 litre air samples

Upper detection limit for air sampler = 258 colonies

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