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ANTI-MICROBIAL EFFICACY OF SAN-AIR GEL

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Executive summary:

We tested the ability of San-Air Mould Gone bio-clean gel to reduce the numbers of airborne coronavirus, bacteria (*Escherichia coli*) and mould spores (*Aspergillus flavus*). The tests have been conducted using a Bacterial Filter Efficiency Test Rig (CH Technologies, USA) and an Anderson device.

An initial test using the San Air gel suspended in media and exposed to the mouse coronavirus MHV-1 (an approved surrogate for human SARS-CoV-2, the cause of the COVID-19 pandemic), demonstrated that direct contact of the gel reduced the numbers of coronavirus MHV-1 by >99.99% (>4log₁₀ plaque forming units/ mL).

Subsequent tests used MHV-1, *E. coli* K12 and *A. flavus* ATCC 9643 that were aerosolised into a chamber that contained the San Air gel that had been allowed to evaporate into the chamber for 10 minutes prior to the microbial aerosols being introduced. The aerosols of the microbes passed through the chamber and were collected into the Anderson device.

These tests demonstrated that evaporated San Air gel was able to reduce the numbers of coronavirus MHV-1 by ~48%, *E. coli* K12 by ~50% and spores of *A. flavus* ATCC 9643 by ~70%.

The ability of the San Air product to reduce the numbers of coronavirus, bacteria and fungal spores in aerosols demonstrates that it is likely to be able to reduce the numbers of these microbes in aerosols if used in rooms or other areas.



AIM-1: To evaluate the antimicrobial efficacy of San-Air gel against coronavirus.

MATERIALS:

1. MHV-1 (ATCC/VR261) (SARS-CoV-2 surrogate)
2. A9 mouse cells (ATCC CCL 1.4)
3. San Air gel – Air Purifier bio-clean gel
4. Neutralizer – 20% w/v bovine serum albumin
5. Cell culture medium (Dulbecco's Modified Eagle Medium; DMEM)
6. Agar – 2% w/v
7. 12-well tissue culture plates
8. 4% paraformaldehyde solution
9. 0.5% crystal violet stain

METHODS:

The coronavirus Murine Hepatitis Virus (MHV-1; ATCC/VR261) was suspended in cell culture media (DMEM) to a concentration of 10^5 plaque forming units (PFU)/ml. SAN AIR gel (25 or 50 mg) was added to 1 ml of cell culture media (DMEM) and heated at 37 °C in water bath for 10-15 minutes. This solution was then added to the viral suspension in DMEM and the mixture was incubated for 30 minutes or 2 hours (for each amount of gel) at ambient temperature (~21°C). Controls were the viral inoculum incubated for 2 hours without the gel at ambient temperature. Each concentration was tested in duplicate and repeated once.

Following the 30-minute or 2-hour incubation, test and control solutions were added to 20% (w/v) bovine serum albumin (BSA) in phosphate buffered saline and allowed to stand for 10-15 minutes in order to neutralize the active ingredients in the gel. Thereafter, 10-fold dilutions of the viral solutions were performed in 20% BSA and 100 µl aliquots of each dilution were incubated in duplicate in wells of 12 well tissue culture plates containing a monolayer of mouse fibroblast cells (A9; ATCC CCL 1.4). The virus was allowed to adsorb to the cells for 1 hour then an overlay medium (50:50 mixture of 2% (w/v) agar and DMEM) was added to immobilize the virus and restrict virus growth to foci of cells at the sites of initial infection. The mouse cells were then incubated for a further 3 days at 37 °C to allow viral replication and the killing of the underlying cells leading to plaque formation.

After the 3-day incubation, the cells were fixed with 4% paraformaldehyde for 1 hour and stained using crystal violet to visualize the plaques (areas of cell death where the viruses had replicated). The number of plaque forming units (pfu) was enumerated. The \log_{10} reduction was calculated by subtracting the number of plaques in the test wells from the untreated control wells, and percentage reduction was calculated.

RESULTS:

Control wells had $26,750 \pm 1,658$ plaques. A 30 minute exposure to 25 mg of gel reduced the viruses by 98.6% (Table 1). Increasing the exposure time to 2 hours, reduced the number of viruses by 99.2% (Table 1). A 30-minute exposure to 50 mg of the antimicrobial gel was sufficient to kill >99.99% of the viruses (Table 1).

Table 1: Efficacy of San Air Gel against MHV-1 in solution

Sample	Amount of gel (mg)	Exposure time (minutes)	Number of plaques	Log ₁₀ reduction	% reduction
Control	-	-	$26,750 \pm 1,658$	-	-
San Air gel (n=2)	25	30	375 ± 35	1.9	98.598
San Air gel (n=2)	25	120	225 ± 177	2.1	99.159
San Air gel (n=2)	50	30	0 ± 0	4.4	99.996
San Air gel (n=2)	50	120	0 ± 0	4.4	99.996

AIM-2: To evaluate antimicrobial efficacy of evaporated San-Air gel against coronavirus aerosols.

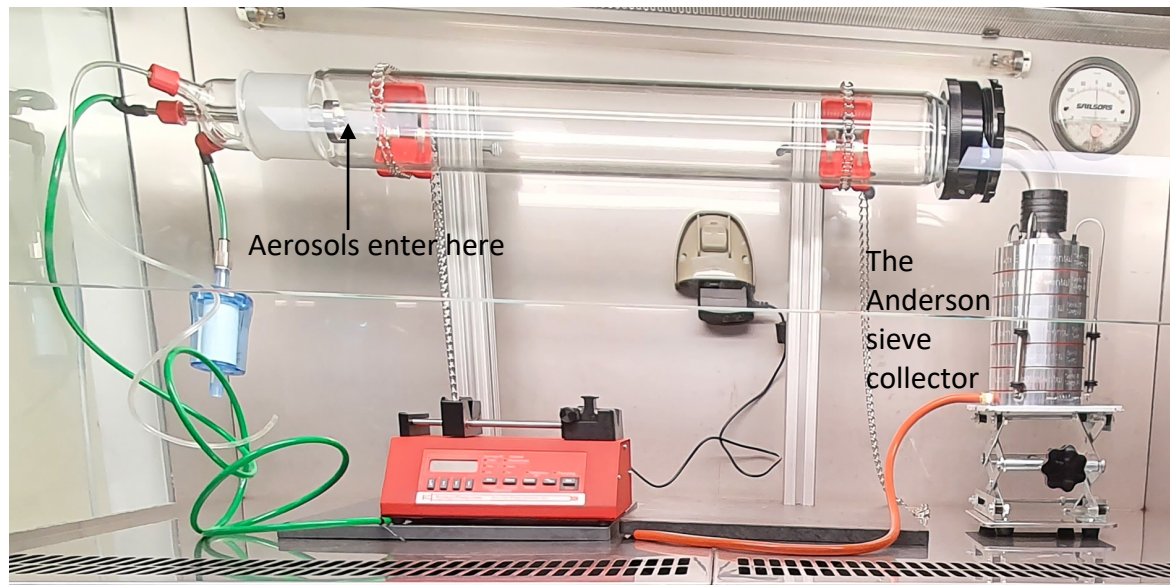
MATERIALS:

1. MHV-1 (ATCC/VR261) (SARS-CoV-2 surrogate)
1. A9 mouse cells (ATCC CCL 1.4)
2. San Air gel – Air Purifier bio-clean gel
3. The Bacterial Filter Efficiency (BFE) Test Rig
4. Cell culture medium (Dulbecco’s Modified Eagle Medium; DMEM)
2. Agar – 2% w/v
3. 12-well tissue culture plates
4. Neutralizer – 20% w/v bovine serum albumin
5. 4% v/v paraformaldehyde solution
6. 0.5% w/v crystal violet stain

METHODS:

The Bacterial Filter Efficiency (BFE) Test Rig (CH Technologies, USA) was used to produce viral aerosols (Figure 1).

Figure 1: The Bacterial Filter Efficiency and Anderson sieve sampler.



The BFE Test Rig consists of a glass aerosol chamber, a 4-jet Blaustein Atomizer (BLAM) nebulizer nozzle, syringe pump, 6-stage viable cascade impactor (Anderson sieve collector; Figure 1) and a rotary vane vacuum pump. The viral inoculum (approximately 1.0×10^6 pfu/ml) was aerosolized using a continuous drive syringe pump set to deliver $50 \mu\text{L}$ of the inoculum for one minute through a nebulizer with an airflow of 28.3 L min^{-1} . The nebulizer produces a mean particle size of $3.0 \pm 0.3 \mu\text{m}$ which travels through the glass aerosol chamber and into the Anderson sieve sampler which contains agar plates to collect viable viruses. The 6-stage Anderson sampler uses perforated plates with progressively smaller holes at each stage, allowing particles to be separated according to size; the particle cut-off size for the first stage is $7 \mu\text{m}$ and $0.65 \mu\text{m}$ for the last stage. The air-flow was cut-off after 1 minute to stop aerosolization, however the vacuum pump was allowed to run for a further 1 minute to collect residual aerosols from the glass chamber.

After 2 minutes, the agar plates were removed, 1.5 ml of 20% w/v bovine serum albumin solution was added and the agar surface scrapped using a sterile scraper to collect the viruses. 100 μl aliquots of the scraping from each agar plate was inoculated in duplicate into wells of 12 well tissue culture plates containing a monolayer of A9 mouse fibroblast cells to allow viral infection as described in the previous section.

Control runs were performed at the beginning of each experiment prior to the addition of the San Air gel in the glass aerosol chamber. The test run was conducted after the gel (10 g) was allowed to evaporate into the glass aerosol chamber at ambient temperature for 10 minutes prior to exposure to the viral aerosols. Test and control runs were conducted in duplicate and repeated once. The number viruses from each of the 6 plates per run was enumerated and added to calculate the total viral numbers.

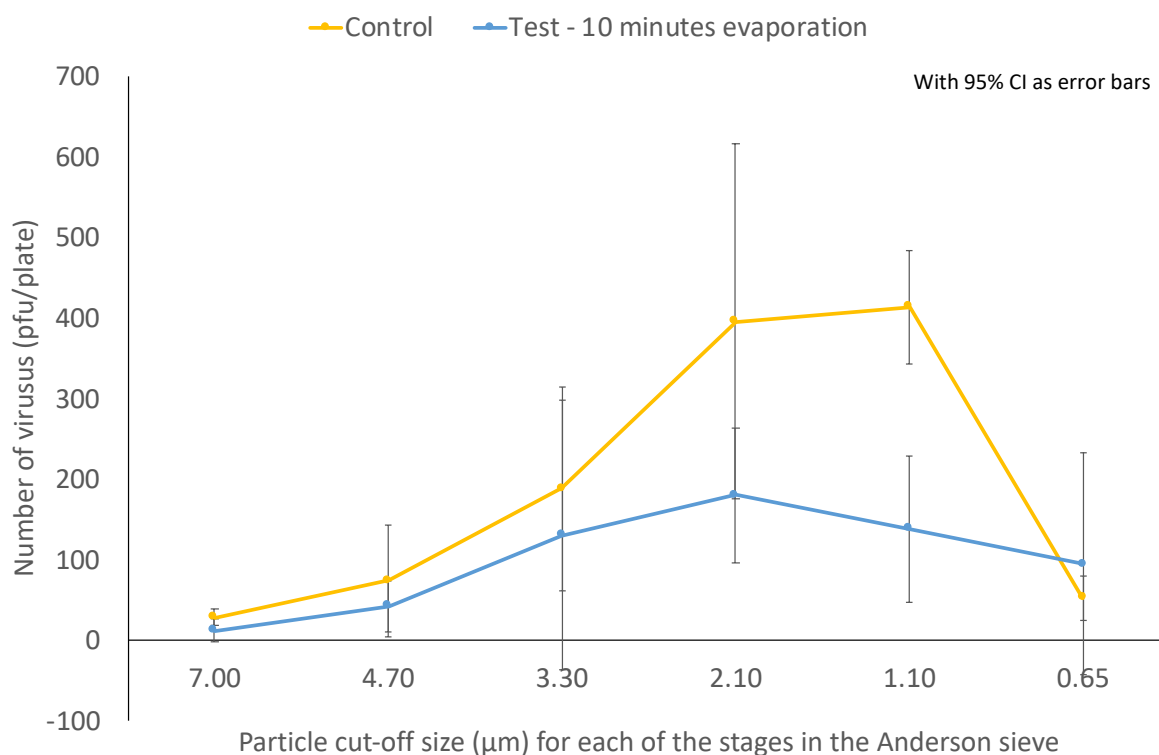
RESULTS:

There were $3.1 \pm 2.5 \log_{10}$ virus in the control run plates (Table 2). Leaving the gel into the glass chamber for 10 minutes reduced the number of viable virus in the aerosol by 45% compared to the untreated controls.

Table 2: Ability of vaporised San Air gel to reduce the number of aerosolised viable coronavirus particles

Sample	Amount of gel (g)	Exposure time (minutes)	Number of plaques	Log ₁₀ reduction	% reduction
Control	-	-	1,152 ± 354	-	-
San Air gel (neutralized)	10	10	596 ± 149	0.3	48.264

Figure 2: The effect of San Air gel on the number of viral particles removed from each of the agar plates in the Anderson sieve



As can be seen in Figure 2, in the absence of San Air in the chamber, the viral particles travelled predominantly in the 3.30 to 0.65 µm aerosols, with most being in the 2.10 and 1.10 µm aerosols. When the San Air gel was allowed to evaporate for 10 minutes in the chamber, the numbers of viral particles that were able to infect the mouse cells was reduced in most aerosol sizes, with a significant reduction in the 1.10 µm aerosol ($p < 0.05$) of 66.667%.

AIM-3: To evaluate antimicrobial efficacy of San-Air gel against bacterial aerosols.

MATERIALS:

1. *Escherichia coli* K12

2. San Air gel – Air Purifier bio-clean gel
3. Bacterial Filter Efficiency (BFE) Test Rig
4. Tryptone soy agar (TSA; Oxoid, Basingstoke, UK)
5. Tryptone soy broth (TSB; Oxoid, Basingstoke, UK)
6. Neutralizer – TSA containing lecithin & Tween 80 (TSAN)
7. Phosphate buffered saline pH 7.4 (PBS; NaCl 8 g L⁻¹, KCl 0.2 g L⁻¹, Na₂HPO₄ 1.15 g L⁻¹, KH₂PO₄ 0.2 g L⁻¹)

METHODS:

Bacteria were grown overnight in TSB and re-suspended in PBS (approximately 1.0 x 10⁴ colony forming units [CFU]/ml). Aerosols were generated as described in the previous section. TSA or TSAN plates were used to collect viable bacteria in the Anderson sieve (see above) to compare the effect of neutralization of the active ingredients in the San Air gel. Control runs were conducted prior to the test runs. Test runs were conducted after the antimicrobial gel was left in the glass aerosol chamber for 10 minutes. Test and control runs were conducted in duplicate and repeated twice.

At the end of each run, the agar plates were incubated at 37 °C for 24 hours, the number of colonies on each of the 6 plates per run were enumerated and the total number of bacterial colonies calculated.

RESULTS:

There were 113 ± 42 CFU of *E. coli* K12 on the control plates with or without the neutralisers. Allowing the gel to vaporise in the glass chamber for 10 minutes and collecting the bacterial cells on plates that did not contain the lecithin or Tween 80 neutralisers reduced the number of viable bacteria in the aerosol by 51% compared to the untreated controls (Table 3). When the experiment was run but the neutralizers were added to the agar, there was a reduction of 29% in the bacterial counts (Table 3).

Table 3: The ability of vaporised San Air gel to reduce the numbers of aerosolised *E.coli* K12 aerosols

Sample	Amount of gel (g)	Exposure time (minutes)	Number of bacteria	Log ₁₀ reduction	% reduction
Control (no neutraliser)	-	-	86 ± 14	-	-
San Air gel (no neutraliser)	10	10	42 ± 20	0.3	51.163
Control (plus neutraliser)	-	-	139 ± 46	-	-

San Air gel (plus neutraliser)	10	10	99 ± 47	0.1	28.777
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Figure 3: The effect of San Air gel on the number of *E. coli* K12 cells collected from each of the agar plates in the Anderson sieve

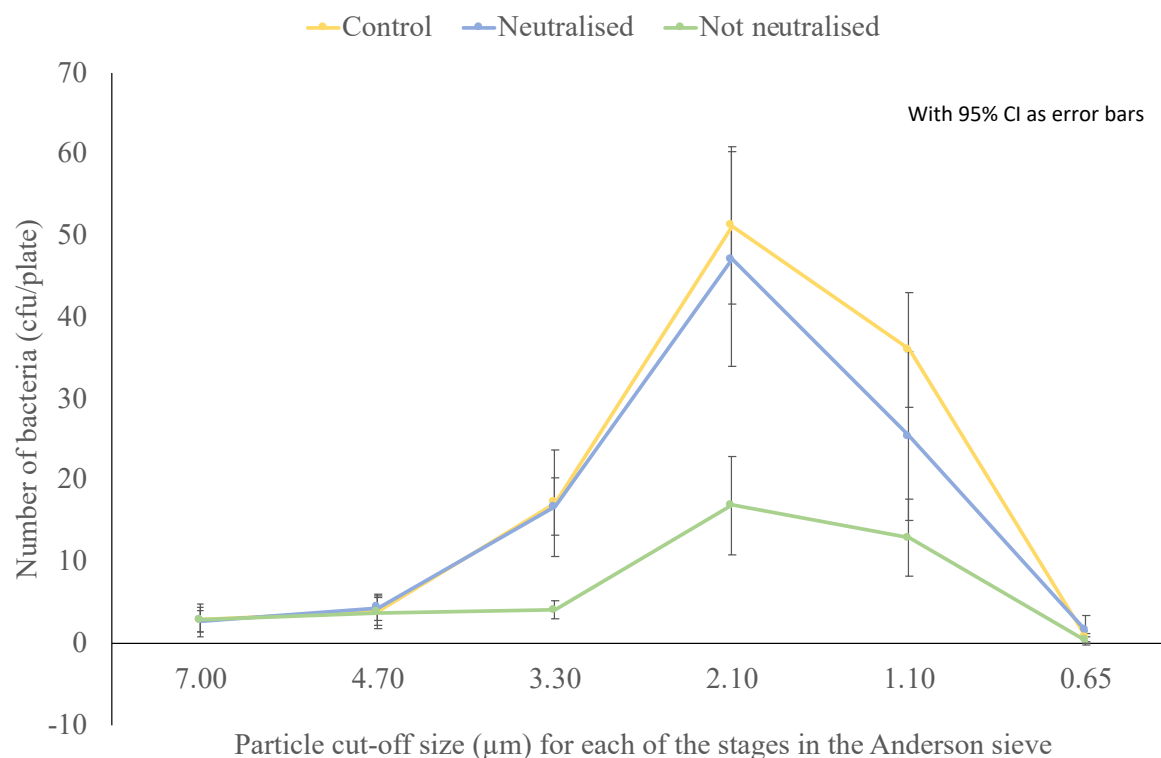


Figure 3 shows the aerosol particle size that the *E. coli* travelled in was predominantly 3.30 to 1.10 µm in the absence of San Air gel. In the presence of San Air gel and without neutralising the effect of the gel whilst the bacteria were growing, there was a significant ($p < 0.05$) reduction in viability of *E. coli* cells travelling in the 3.30, 2.10 and 1.10 µm aerosols of 75.962, 66.883 and 63.972% respectively. If the San Air gel was neutralised during the growth of the *E. coli* there was still some reductions in viability, but this did not reach significance compared to the control without San Air gel.

AIM-4: To evaluate antimicrobial efficacy of San-Air gel against aerosolized fungal spores.

MATERIALS:

1. Aspergillus flavus ATCC 9643 spores
2. San Air gel – Air Purifier bio-clean gel
3. The Bacterial Filter Efficiency (BFE) Test Rig
4. Sabouraud’s dextrose agar (SDA; Oxoid)
5. Neutralizer – SDA containing lecithin & Tween 80 (SDAN)

METHODS:

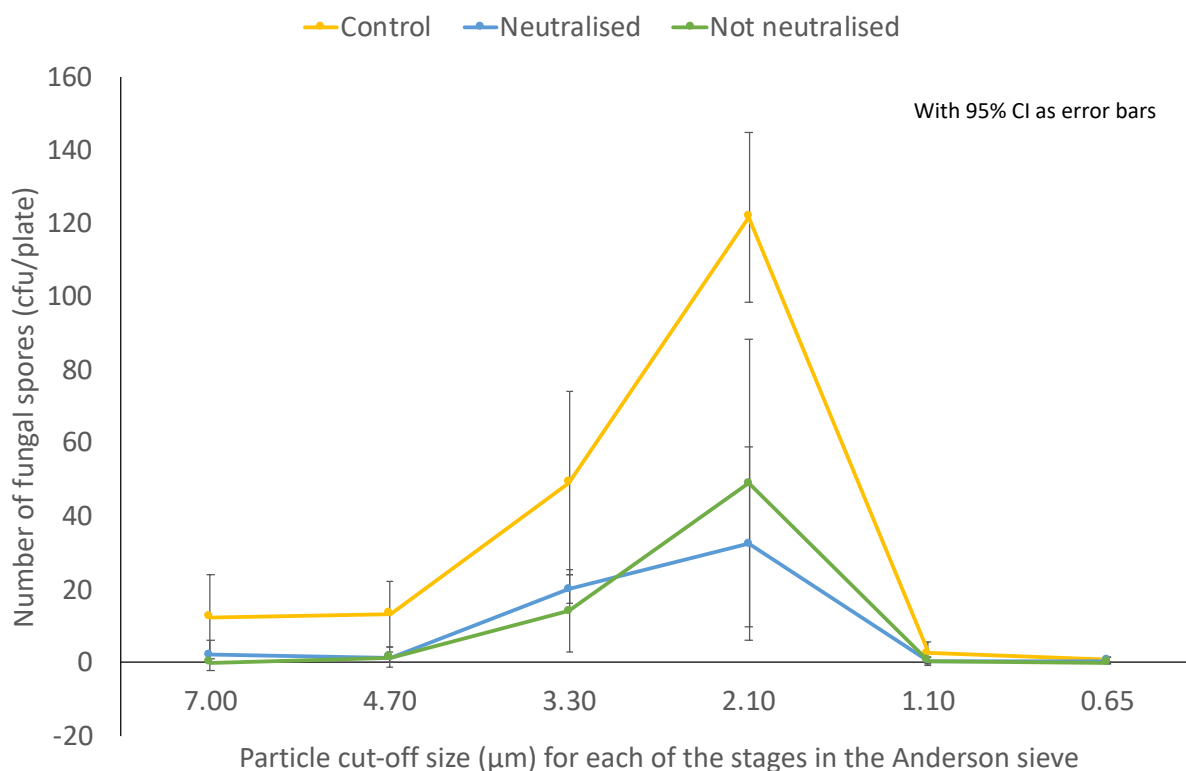
Fungi were grown on SDA plates at 25 °C for up to 5 days to allow spore formation. The fungal cells were scrapped from the agar and suspended in sterile deionized water. The suspension was filtered through sterile 70 µm filters to remove hyphal fragments. The spores were re-suspended in sterile deionized water to a concentration of approximately 1.0×10^4 cells/ml. Aerosols were generated and viable spores collected on SDA/SDAN plates as described above. Agar plates were incubated at 25 °C for 48 hours. The number of colonies on each of the 6 plates per run were enumerated and added to calculate the total number of fungal colonies.

RESULTS:

There were on average 200 ± 62 CFU on the control plates. Leaving the antimicrobial gel into the glass chamber for 10 minutes reduced the number of viable fungi in the aerosol by 72% compared to the untreated controls (Table-4). The addition of neutralizers to the agar reduced the fungal counts in the test samples compared to controls by 67%.

Sample	Amount of gel (g)	Exposure time (minutes)	Number of fungi	Log ₁₀ reduction	% reduction
Control (no neutralizer)	-	-	231 ± 42	-	-
San Air gel (no neutralizer)	10	10	65 ± 7	0.6	71.861
Control (neutralized)	-	-	170 ± 77	-	-
San Air Gel (neutralized)	10	10	57 ± 23	0.5	66.471

Figure 4: The effect of San Air gel on the number of *A. flavus* spores collected from each of the agar plates in the Anderson sieve



In the absence of the San Air gel, the spores of *A. flavus* travelled in the 7.00 to 2.10 µm aerosols, with virtually no spores travelling in the 1.10 or 0.65 µm aerosols (Figure 4). The addition of the San Air gel to the BFE resulted in a reduction in numbers of spores that remained able to germinate into hyphae, with a significant reduction in the 2.10 µm aerosols ($p < 0.05$) of 66.530%. There was no effect of neutralisation of the San Air on the numbers of spores remaining viable.

CONCLUSIONS:

- San Air gel vapours are able to significantly reduce
 - the numbers of coronaviruses that can infect cells
 - the number of *E. coli* cells
 - the number of *A. flavus* spores that can germinate
- San Air gel vapours are bacteriostatic, as they are more active if they are not neutralised whilst the bacteria (*E. coli*) are growing
- San Air gel vapours are fungi-sporicidal as there is no effect on neutralisation on the germination of the spores
- **Next steps:**
 - Repeat once more the *A. flavus* spore test
 - Evaluate the effect of not adding a neutraliser on viricidal activity
 - Evaluate allowing the San Air to evaporate for 20 mins in the chamber.