A microbiological assessment of aerosol generated during debond of fixed orthodontic appliances

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Keywords: bio aerosol, particles, dental

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Abstract

Introduction - The aims of this study were to describe bacterial load and diversity of the aerosol created during the process of enamel clean up following the removal of fixed orthodontic appliances and to assess the effect of a pre-procedural mouthrinse.

Methods - The study involved the sampling of ambient air adjacent to the patient's mouth during the process of adhesive removal using a slow speed handpiece and a spiral fluted tungsten carbide bur without water irrigation. Sampling was carried out during enamel clean up with or without a pre-procedural mouthrinse of either sterile water or chlorhexidine. Airborne particles were collected using a viable inertial impactor simulating the human respiratory tree. The bacteria collected were analysed using both culture and molecular techniques.

Results - Bacteria produced during debond and enamel clean up can reach all levels of the respiratory tree. The use of a pre-procedural mouthrinse, either sterile water or chlorhexidine, increased the number and diversity of the bacteria in the air.

Conclusions – When using a slow speed handpiece and spiral fluted tungsten carbide bur for enamel clean up after orthodontic treatment, the bacterial load and diversity, of the aerosol produced, is less when a pre-procedural mouthrinse is not used.

Keywords: bio aerosol, particles, dental

Introduction

Within the dental clinic a number of different instruments are capable of generating aerosol particulates, *i.e.* those with aerodynamic diameters of 50µm or less[1]. These include air turbines, ultrasonic scalers[2] and slow speed hand pieces, all of which may be used during enamel clean up after orthodontic debond. Whereas large particles created during their use may show a ballistic trajectory and fall quickly to the floor or other nearby surface, smaller diameter particles have the potential to remain airborne for extended periods and therefore be inhaled by dental operators, assistants or patients.

Once inhaled, the level at which these particles settle within the respiratory tree will depend on factors such as the lung anatomy[3], nasal or oral breathing pattern, the presence of respiratory disease, particle size and particle density[4,5]. Although many will settle superficially in the conducting airways and terminal bronchi, smaller particles may impact as deep as the terminal alveoli[6] and so may be retained within the lung for long periods before being cleared by the alveolar macrophages[7].

In addition to solid particulates created during operative dental procedures, liquid aerosols may also be created. Such aerosol droplets may contain solid particulates as well as microbial contaminants, *e.g*. bacteria and fungi, in which case they are often referred to as bio aerosols. The dynamics of aerosol behaviour are complex and dependent on factors such as liquid composition and environmental factors, including temperature, humidity and air flow. Previous work has shown that bio aerosols have considerable potential for infection of human hosts when the droplet diameter is in the region of 0.5 to 20µm[8].

Interestingly within dentistry, orthodontists perceive themselves to be at less risk of contracting disease from their patients than general dental practitioners, probably because their patients tend to be predominantly children and young adults[9]. However, perhaps as a consequence of this perceived lower risk, orthodontists are less likely to adhere to infection control procedures[10]. This is despite the fact that within 5 minutes of beginning enamel

clean up at orthodontic debond there is a significant increase in bio aerosols compared with pre-operative resting room levels[11]. The aerosol particles liberated into the working atmosphere contain micro-organisms derived from the patient's oral cavity.

Although microbiological culture techniques have previously been used in the examination of dental aerosols, approximately 50% of intraoral microbes cannot be cultured in the laboratory. Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE)[12] techniques can be used to identify further microbial species, but to date there are no published studies employing PCR and DGGE techniques in the microbiological analysis of dental or orthodontic aerosols.

The aim of the present study was to investigate the bioaerosols created during the process of debonding and enamel clean up following orthodontic fixed appliance therapy. The specific objective was to investigate the effect of pre-procedural rinsing prior to debond, with either water or Chlorhexidine gluconate, on the bacterial load and biodiversity of the aerosol produced.

Materials and Methods

18 consecutive patients who were undergoing routine debond of their upper and lower fixed orthodontic appliances followed by enamel clean up were recruited into this study. Eligible patients were those who had undergone a course of comprehensive fixed appliance treatment, *i.e.* upper and lower arches were treated to completion and the patient was scheduled for debond of the fixed appliances. Patients undergoing single-arch or sectional appliance treatment were excluded, as were those who were undergoing or who had recently undergone a course of antibiotic treatment. Selection for inclusion in the study was on a consecutive basis, providing eligibility criteria were met. No age restrictions were set, nor were there maximum or minimum limits on treatment time. The patients were divided into three groups of six, with each group being allocated to a particular method of composite removal at appliance debond, namely:

1. Slow speed hand piece, no pre-procedural mouth rinse
2. Slow speed hand piece, 0.2% Chlorhexidine gluconate pre-procedural mouth rinse
3. Slow speed hand piece, sterile water pre-procedural mouth rinse

All six patients in a group were sampled before proceeding to the next group.

Formal ethical committee approval was not required as this was not deemed an interventional study, but a study of air quality. All three composite clean up methods were already in routine use at debond within the orthodontic department. Prior to debond each patient's oral hygiene status was recorded using the Index of Oral Cleanliness, a 5-point scale proposed by Bearn *et al.* (1996)[13].

Air sampling was carried out in the orthodontic department of Musgrove Park Hospital (Taunton, Somerset, UK) using a Thermo-Electron 10-800 6-stage viable particle sampler (Thermo Fisher Scientific Inc, USA) (Figure 1). This type of impactor has been in use since the early 1980's[14] and comprises six impactor stages which correspond to various levels within the human respiratory tree as shown in Figure 2.

Each of the six stages of the impactor contained a glass Petri dish within which there was a solid fastidious anaerobe agar (FAA) containing 5% defibrinated horse blood. Attached to the top of the impactor at the inlet port was an extension tube used to simulate the pharynx. Attached to the bottom of the impactor was a vacuum pump used to draw the air through the impactor stages. The air flow through the impactor was set at 28.3 l/min according to the manufacturer's instructions. The air intake of the extension tube was positioned so that it was level with the patient's mouth at debond and at a distance of 30cm.

In patient groups 2 and 3 the patient was initially asked to perform a pre-procedural mouth rinse. The pre-procedural Chlorhexidine and water mouthwashes were dispensed via plastic cups in volumes of 15ml and patients were asked to rinse thoroughly for a period of one minute, just prior to bracket removal.

In each of the three groups the upper and lower fixed appliances were removed using debanding and debonding pliers as is normal practice. Following this the impactor was allowed to run for 1 minute prior to composite removal. Air sampling was then carried out for 15 minutes during the composite removal procedure. If composite removal was completed in less than this time the impactor was still allowed to run for the full 15 minutes. In each case composite removal was performed using a spiral fluted tungsten carbide bur in a slow speed handpiece, without water coolant, but with a high volume chair side aspirator held close to the patient's mouth as is normal practice. For all patients the composite that had been used to bond the brackets to the teeth and was therefore being removed at debond was Transbond XT (3M Unitek, Monrovia, USA). The band cement was Intact Glass Ionomer Cement (Orthocare, Bradford, UK).

Following debond, composite removal and sampling, the six glass Petri dishes were removed from the impactor, covered and transferred to an anaerobic incubator for seven days at 37^{o}C. Following this incubation period the dishes were removed and under good illumination were examined for microbial growth on the agar surface. For each Petri dish the colony count and number of different colony types was noted, along with the total number of colony forming units for each type. In distinguishing one colony type from another, factors such as size, colour, surface texture, dryness/wetness and the contour of colony margins were examined with the naked eye.

Finally, the total bacterial growth present on each plate was collected by washing with 1.5 mL Tris-HCl/EDTA buffer solution, and sweeping the surface with sterile plastic loops to collect all adherent colonies. This plate sweep sample was pipetted into 2.0mL Eppendorf containers and stored at -80^{o}C in a laboratory freezer to be retained for later molecular analysis.

The molecular techniques applied to each plate sweep sample were:

a) Extraction of DNA

b) ‘Universal’ 16S rDNA-directed PCR of the extracted DNA, using a thermal cycler

c) Agarose Gel electrophoresis of the PCR product

d) DGGE of the PCR product

Once each Petri dish had been swept for bacterial growth, the growth medium was removed and the dish thoroughly cleaned and sterilised prior to re-use.

DNA extraction - For each patient sample, six Eppendorf tubes were removed from the freezer and left to defrost on the laboratory workbench at room temperature for 20 minutes. After defrosting the GenElute^{TM} kit (Sigma Aldrich, USA) was used to extract the bacterial DNA. The sample was subjected to a number of standardised chemical processes, designed to extract the DNA from Gram positive bacterial cells. In summary, these were:

1. Bacterial breakdown to release DNA
2. Preparation of a DNA-binding membrane.
3. Transfer of the bacterial lysate through the membrane, to retain the DNA.
4. Washing of the membrane to release the DNA.

The different stages involved were performed according to the manufacturer’s instructions and all chemicals required were included in the standard kit, as supplied. Other materials necessary were routine items of equipment found in most microbiology laboratories, including a centrifuge, hot water baths and whirlimixer. The end result of this process was a solution containing the collected bacterial DNA of all species for each single Petri dish sweep sample.

Polymerase Chain Reaction (PCR) - A PCR stage was required to amplify the extracted DNA to a level detectable by DGGE. For a set of six DNA samples corresponding to collected bacteria at each of the six impactor stages, a stock solution was prepared consisting of the following:

1. PCR water (distilled) 88.5µL
2. Green buffer 60µL
3. Forward primer 30mM 12µL (27F: AGA GTT TGA TCC TGG CTC AG)
4. Reverse primer 30mM 12µL (1492R: TAC GGG TAC CTT GTT ACG ACT T)
5. dNTP mix 6µL
6. Taq polymerase 1.5µL

This was dispensed into 6 small Eppendorf tubes in volumes of 30µL, labelled to indicate impactor stages one to six, and an extra seventh tube was prepared with the above components in the same proportions. To the seventh tube, 20µL of distilled PCR water was added and all seven tubes subjected to one minute of ultraviolet light exposure within a hooded ultraviolet light transilluminator. This stage was carried out to eliminate any viable DNA which may have contaminated the Eppendorf tubes during the preparation process. To the first six tubes, 20µL of DNA was added from each of the six impactor stage samples, while the extra seventh tube was retained as a negative control. The seven Eppendorfs were placed within a PCR thermal cycler and subjected to the following program:

- predenaturation step of 94°C for 2 minutes
- 34 cycles of 94°C for 30 seconds, 56°C for 60 seconds, 72°C for 2 minutes
- final extension step of 72°C for 10 minutes
- temperature held at 10°C

To confirm successful DNA extraction the amplified PCR product was run on an agarose gel.

Agarose Gel - A 1.2% concentration agarose gel was prepared by adding 1.2g of agarose powder to 100ml of TBE in a 100ml beaker and heating the mixture in a microwave oven for 2 minutes at 800W. The beaker was then placed on a magnetic stirrer and allowed to cool until it had begun to thicken. At this point, 3µL of ethidium bromide was added and allowed

to disperse under the action of the magnetic stirrer. An agarose gel casting kit tray was prepared according to the manufacturer's instructions, with a comb insert creating sample wells. This was removed after cooling.

An agarose marker solution was created by adding 15µL of a 100 base-pair ladder to 50µL of loading dye, and 6µL of this was pipetted into the first well, this also acted as a positive control. Into adjacent empty wells, 10µL of the PCR product for each impactor stage was pipetted, carefully recording the position of each sample within the gel. The gel tray was then seated within an electrophoresis cell and the tank filled with TBE buffer. Once immersed, the tank was closed and 120v electrical potential was applied for 45 minutes across the gel.

Removing the gel from the tray and exposing it to ultraviolet light in the transilluminator allowed confirmation of a positive PCR product by noting the presence of dark bands distant to the site of the sample wells. Image capture was performed using the DigiDoc-It Darkroom bioimaging system (UVP, Upland, USA).

Denaturing Gradient Gel Electrophoresis (DGGE) - Following confirmation of PCR product, the DGGE step was required to identify the number of different bacterial species present within a sample obtained from each stage in the impactor. This required the use of a gel chamber into which two solutions would be combined as liquid, but would set to form a 0.75mm thick rectangular gel, in which the concentration of urea formed an increasing gradient from top to bottom.

The solutions required were prepared as follows:

Solution A:

- 11.5ml 70% DGGE denaturant (stock solution of 18.8ml 40% Acrylamide, 2ml 50x TAE buffer, 16ml Formamide, 16.8µL urea, made up to 100ml with distilled water)
- 80µL APS

- 5µL TEMED

Solution B:

- 11.5ml 40% DGGE denaturant (stock solution of 18.8ml 40% Acrylamide, 2ml 50x TAE buffer, 28ml Formamide, 29.4µL urea, made up to 100ml with distilled water)
- 80µL APS
- 5µL TEMED

The gel was prepared by the addition of solutions A and B to the two separate chambers of a gradient maker, which controlled the ratio of one solution to the other and allowed a steadily changing mixture of the two to flow into the glass plate assembly via a blood collection kit. Throughout this process, a magnetic stirrer ensured that the A and B mixture was homogenous. The combined denaturants were run into the gel chamber until the top had been reached. A plastic comb was inserted into the liquid, which would form a number of wells once the gel had set. The gel was allowed to set for 20 minutes.

Once set, the comb was removed to expose a number of open wells on the upper edge of the gel. For each of the samples, 6µL of PCR product was combined with 6µL of DGGE loading dye. 10µL of this mixture was pipetted into an empty well at the top of the gel, and the position of each sample recorded. The remaining 2µL of the mixture allowed for a margin of pipetting error.

The gel chamber was lowered into the DGGE-2401 electrophoresis tank, containing 22 litres of Tris-acetate EDTA (TAE), maintained at 60^{o}C, and 220v applied through the gel for a period of four hours. Once complete, the chamber was removed and disassembled.
A solution of Sybr Green staining reagent in 1x TAE was prepared in the ratio of 15µL Sybr Green to 150ml TAE. The DGGE gel was transferred from the gel chamber to a plastic bath containing this staining solution, which was then placed on an oscillating rocker table for 20 minutes. After 20 minutes of gentle agitation, the staining stage was complete and the gel transferred to the transilluminator and exposed to transilluminated ultraviolet light. Once a

sequence of bands within the gel had been visualised, the Kodak Digital Science electrophoresis documentation and analysis system (Rochester, USA) was used to record the resultant image.

On each image the number of unique bands (and therefore the number of different bacterial species) per impactor stage was noted, providing data on the biodiversity of samples obtained under the four different sampling conditions.

Data analysis was undertaken on a descriptive basis. To describe bacterial load and biodiversity means are standard deviations are provided.

Results

6 patients were recruited to each of the 3 debonding groups (no rinse, chlorhexidine rinse and water rinse) with each patient having 6 samples, one for each of the impactor cut-off stages. With respect to the Index of Oral Cleanliness, as might be expected of patients undergoing orthodontic treatment, there were very few patients scoring highly, *i.e*. poor standard of oral hygiene. Most patients scored 0, 1 or 2, with only one patient having a score of 4 and this patient underwent a no pre-procedural rinse debond.

Bacterial load – Table 1 summarises the bacterial load for each of the 6 impactor stages at background level and in each of the 3 patient groups. In all 3 groups total bacterial count was higher than at background level. For each group and at background, the lowest count was recorded at impactor stage 6 (corresponding to the level of the respiratory alveoli) and the highest levels noted from levels 2 to 5 (corresponding to the pharynx down to the terminal bronchi respectively). Pre-procedural rinsing with either chlorhexidine gluconate or water was associated with an increase in bacterial load.

Biodiversity - The biodiversity is represented by the number of different bacterial colony types cultured or by the number of bands visible on a DGGE gel. The number of morphologically distinct bacterial colony types was higher with Chlorhexidine or water pre-rinse compared to no pre-procedural rinse (Table 2). Molecular techniques (PCR and DGGE) identified 5 to 6 times the number of bacterial species than the culture method. However, the same trends were identified for the effect of pre-procedural rinsing (Table 3). For both identification techniques the lowest diversity was observed at impactor stage 6, equivalent to the level of the terminal alveoli in the respiratory tree. An example of a DGGE gel showing the PCR amplicons can be seen in Figure 3.

Discussion

The patients selected to take part in this study were consecutive routine debonds at the orthodontic Department Musgrove Park Hospital, Taunton and the methods of debond and clean up were variously used by different operators in the department. Sampling of eligible patients was performed until six patients had been sampled in each of the three test groups.

The sampling distance of 30cm from the mouth was chosen as it is representative of the distance that the orthodontist's mouth and nose are from the patient's mouth during operative procedures. This distance has also been used in other orthodontic particulate studies and for the same reasonr[15].

As far as possible attempts were made to control the atmospheric sampling conditions from one debond to the next. This was achieved by carrying out the debonds in a side surgery, separate from the main clinic. Doors and windows remained closed and no air-conditioning or fan units were in operation. Despite these measures, it is likely that some inconsistencies would occur in terms of air turbulence and human activity, as the environmental conditions were only tightly controllable during the sampling time. It is

possible that uncontrolled atmospheric changes outside of the sampling time may have had an effect on the aerosol sampled during debond. This is because aerosol particles may remain airborne for extended periods of time, with some very small particles able to remain suspended for several days in non-turbulent air[16].

Bacterial load and pre-procedural rinsing - In this study a marked increase in bacterial load was observed during debond and enamel clean up, particularly when compared with background air samples where there was no clinical activity. This is perhaps not unexpected, given that contaminated resin particulates and aerosol droplets will be liberated from the mouth into the surrounding air around the patient, and is consistent with the findings of previous workers who demonstrated statistically significant differences between clinical and non-clinical samples[11].

However, what was interesting from the results of this present study is that the bacterial load of the sampled air at debond increased following the use of a pre-procedural mouth rinse, whether it was sterile water or chlorhexidine. This is contrary to the findings of other related studies. In one such study on air polishing of teeth, the use of a pre-operative chlorhexidine rinse caused a significant reduction in bacterial numbers compared with the controls who had rinsed with distilled water[17]. Previous work by Toroglu *et al*. (2001)[11] comparing high-speed water-irrigated debonds with and without a pre-procedural chlorhexidine mouthrinse also found a slight reduction in bacterial numbers. It is known that gram positive bacteria are more susceptible to the anti-microbial effects of chlorhexidine than gram negative species and that the oral cavity has a high proportion of gram positive bacteria. Therefore in the present study it might have been expected that the use of chlorhexidine at least would have reduced the number and viability of aerosolised bacteria during orthodontic debond. A possible explanation for our finding of more bacterial colony units may lie in chlorhexidine's mode of action, which acts to loosen plaque from tooth surfaces. In addition, because of differences in composition, viscosity and surface tension

between chlorhexidine and saliva, chlorhexidine droplets may be more easily displaced from the tooth and therefore aerosolised. Together, these two phenomena may potentially have lead to an increase in the number of colony forming units observed.

The present study also showed that the use of a sterile water pre-procedural rinse produced higher numbers of bacteria than in the non-rinse group. By contrast, two previous studies demonstrated a mean bacterial reduction of between 33.9% and 61% when using a pre-operative water mouthrinse[18,19]. In the absence of any possible pharmacological effect, it is possible that in both cases the reduction in bacterial numbers was due to the simple mechanical cleansing action of the liquid. Although the duration of mouthrinsing in the present study was double that of the earlier research (30 seconds), similar reductions in the number of colony forming units (CFU) were not observed. Instead there was a modest increase in the mean number of CFU, compared with the non-rinsing group.

Bacterial load and diversity at different impactor stages - The design of the viable impactor enables the separation of airborne aerosols and their accompanying bacteria according to aerodynamic diameter. The results of this study demonstrate that the aerosol and therefore bacteria produced during debond and enamel clean up have the potential to reach all parts of the human respiratory tree, including the deepest terminal alveoli. Although the number of bacteria that may potentially reach the deepest parts of the lung was less than elsewhere, there was no consistent gradient from the upper to the lower stages of the impactor and therefore upper to the lowest parts of the respiratory tree. A similar finding was found by Day *et al*. (2008)[6] when looking purely at particulate rather than bacterial aerosol production during enamel clean up at debond.

It is known that the oral cavity contains a large number of different bacterial species, with over 500 having been reported in the literature[20] and that the relative proportions of these species are not equal from one oral site to another[21,22]. In the present study it is likely

that a major source of the aerosolised bacteria were from supragingival plaque on the tooth surface. An unknown proportion is also likely to be derived from aerosolised saliva within the mouth. In a comprehensive study of multiple colonised sites, between 11 and 52 individual bacterial species have been reported as being present in supragingival plaque samples when using molecular detection techniques[22]. Similarly up to 53 different DGGE amplicons and therefore bacterial species have previously been identified in a single saliva sample[23]. In the present study a maximum of 40 different DGGE bands were detected in a single sample of bioaerosol at debond. This smaller number may be in part due to the indirect nature of the aerosol sample collection. In addition, whilst sampling was being carried out, a high volume aspirator was also being held in close proximity to the mouth, as a result of the findings of previous studies on respirable particulates at orthodontic debond[6,15].

When bacterial load and diversity in the surgery was measured without any clinical procedures being performed, not surprisingly bacteria were still detected. Previous work has shown that less than 1% of airborne particles are normally contaminated with bacteria[24], but this might be expected to be higher in the dental clinic. This is because respirable particulates may remain airborne for several days in non-turbulent air and many such aerosol particulates are likely to be produced in dental clinical environments during operative procedures.

Two methods were used to assess biodiversity. One was by counting the number of morphologically different colony types present on agar plates, and the other was by counting the number of individual bands produced by DGGE. Counting the cultured colonies suggested that rinsing with either water or Chlorhexidine actually increased biodiversity compared with non-rinsing, as did the results of the PCR/DGGE. In both methods of assessment, the non-clinical sampling group demonstrated the lowest biodiversity.

In all cases the biodiversity of each debond was observed to be greatest at the first impactor stage and least at the sixth stage, corresponding to the deepest parts of the lung.

It is known that with decreasing particle size (*i.e*. through successive stages within the impactor) the viability of sampled bacteria decreases[24], further reducing the potential for biodiversity at deeper impactor stages.

Conclusions

1. Debond and enamel clean up with a slow speed handpiece and a spiral fluted tungsten carbide bur, without irrigation, produced a bioaerosol with the potential to infiltrate the respiratory tree, from the pharynx to the terminal alveoli of the lung.
2. The use of a pre-procedural water or Chlorhexidine rinse appeared to cause an increase in the number and diversity of airborne bacteria.
3. Even in the absence of clinical activity bacteria were still sampled from each of the 6 impactor stages in the dental clinic.

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Figure legends

Figure 1 The Andersen six-stage viable particle impactor assembled and disassembled showing each of the 6 stages and the Petri dishes with agar ready to be loaded.

Figure 2 Cross sectional diagram of assembled six-stage Andersen impactor showing the aerodynamic diameter cut off for each stage in microns along with the corresponding site of impaction in the respiratory system.

Figure 3 An example of a transilluminated DGGE gel showing amplicon bands

Table Legends

Table 1: Mean (SD) bacterial count at each level of impactor for background and each of the 3 treatment groups (Impactor level 6 represents the respiratory alveoli and Impactor level 2 the pharynx)

Table 2: Mean (SD) species count by cultured colonies at each level of impactor in each of the 3 treatment groups (Impactor level 6 represents the respiratory alveoli and Impactor level 2 the pharynx)

Table 3: Mean (SD) number of bands on DGGE gel at each level of impactor in each of the 3 treatment groups (Impactor level 6 represents the respiratory alveoli and Impactor level 2 the pharynx)

Figures

Figure 1 The Andersen six-stage viable particle impactor assembled and disassembled showing each of the 6 stages and the Petri dishes with agar ready to be loaded.

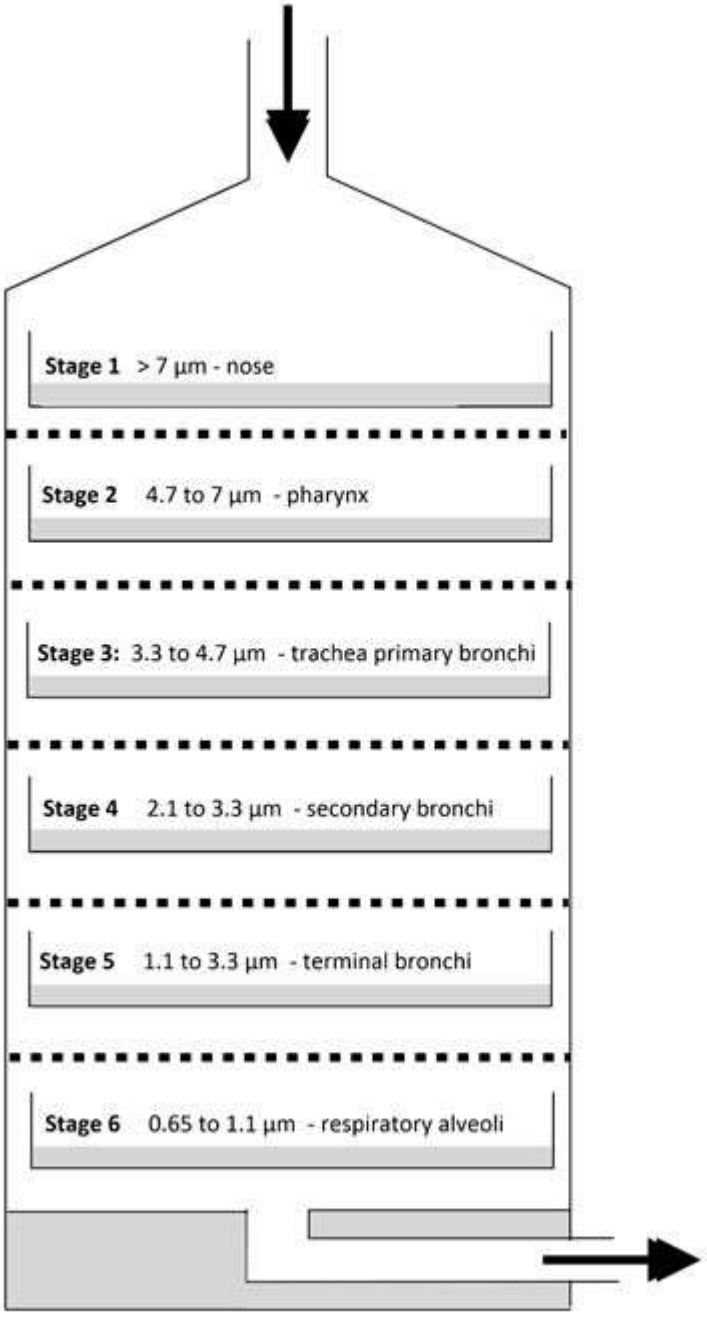

Figure 2 Cross sectional diagram of assembled six-stage Andersen impactor showing the aerodynamic diameter cut off for each stage in microns along with the corresponding site of impaction in the respiratory system.

Tables

	Background			No rinse			Chlorhexidine rinse			Water rinse		
Impactor Level	n	Mean bacterial count	SD	n	Mean bacterial count	SD	n	Mean bacterial count	SD	n	Mean bacterial count	SD
1	6	11.5	2.4	6	12.2	7.6	6	18.2	6.1	6	17.5	17.5
2	6	7.3	2.3	6	11.8	17.1	6	27.7	15.1	6	22.0	18.1
3	6	2.8	2.0	6	14.7	18.9	6	19.8	6.3	6	17.3	15.9
4	6	2.7	1.9	6	7.7	14.9	6	20.8	12.6	6	18.7	11.2
5	6	1.5	1.0	6	10.5	14.2	6	19.5	14.2	6	8.3	7.2
6	6	1.0	0.9	6	6.0	10.6	6	5.3	3.8	6	5.0	3.2

Table 1: Mean (SD) bacterial count at each level of impactor for background and each of the 3 treatment groups (Impactor level 6 represents the respiratory alveoli and Impactor level 2 the pharynx)

	No rinse			Chlorhexidine rinse			Water rinse		
Impactor Level	n	Mean bacterial colonies	SD	n	Mean bacterial colonies	SD	n	Mean bacterial colonies	SD
1	6	4.8	1.2	6	5.2	1.3	6	5.7	2.6
2	6	3.7	1.8	6	6.2	1.7	6	6.2	2.3
3	6	3.8	1.7	6	7.3	1.6	6	6.2	3.3
4	6	2.3	2.5	6	5.2	2.4	6	5.2	2.2
5	6	3.5	1.5	6	5.5	1.8	6	3.7	2.2
6	6	2.0	2.7	6	3.2	1.0	6	2.8	1.6

Table 2: Mean (SD) species count by cultured colonies at each level of impactor in each of the 3 treatment groups (Impactor level 6 represents the respiratory alveoli and Impactor level 2 the pharynx)

	No rinse			Chlorhexidine rinse			Water rinse		
Impactor Level	n	Mean bacterial colonies	SD	n	Mean bacterial colonies	SD	n	Mean bacterial colonies	SD
1	6	23.5	6.6	6	30.5	7.9	6	18.3	4.7
2	6	23.5	4.8	6	31.2	6.1	6	23.8	6.4
3	6	21.7	6.6	6	27.8	4.3	6	21.8	7.4
4	6	19.8	6.6	6	26.5	6.3	6	18	5.4
5	6	20.5	2.1	6	27.5	3.6	6	17	6.4
6	6	17.5	9.2	6	26.0	5.5	6	16.8	8.0

Table 3: Mean (SD) number of bands on DGGE gel at each level of impactor in each of the 3 treatment groups (Impactor level 6 represents the respiratory alveoli and Impactor level 2 the pharynx)