Research Journal of
Microbiology

**Academic
Journals Inc.**

An Artificial Mouth System (NAM Model) for Oral Biofilm Research

Z.H.A. Rahim, A.R. Fathilah, S. Irwan and W.I. Wan Nordini Hasnor
Department of Oral Biology, Faculty of Dentistry,
University of Malaya, Kuala Lumpur 50603, Malaysia

Abstract: The objective of this study was to validate NAM model, an artificial mouth system for use in the study of oral biofilms. The NAM model consists of a cylindrical glass chamber (1×6 cm) which was used to mimic the oral cavity and glass beads (3 mm), placed along its length to provide surfaces for biofilm formation. The opening at both ends of the chamber which were fitted with rubber tubing served as an inlet to and outlet from the system. The two tubing were then connected to a reservoir (bacterial reservoir) via a peristaltic pump. The apparatus was kept at 37°C in a water bath. In the formation of oral biofilm, saliva was first pumped into the system to coat the glass beads. Excess saliva was then rinsed with distilled deionised water. Bacterial inoculum (*Strep. mutans*) was then allowed to flow into the system for 24 h. The bacterial population (cfu mL^{-1}) in the biofilm developed on each of the glass beads in different experiments were analyzed and, validated for reproducibility. Its efficiency in maintaining temperature and flow rate for the experiment and sterility prior to the experiment was also determined. The results obtained showed that the bacterial counts of the biofilms between glass beads are not significantly different (p>0.6) and demonstrated reproducibility (4-5% standard deviation) between different experiments. It was also observed that the flow rate and temperature are constant and sterility of the apparatus is maintained throughout the experiment. This shows that the NAM model is valid for use in the *in vitro* study of oral biofilm development.

Key words: Artificial mouth model, NAM model, oral biofilm, *Strep. mutans*

INTRODUCTION

Since the late 19th century, various artificial mouth models had been invented by researchers around the world (Tang *et al.*, 2003). The curiosity to know and understand what is happening in the mouth has led to the development of artificial mouth models at the laboratory scale. The investigations on oral biofilm are made easier with artificial mouth models which allow for the development of biofilm to be monitored easily by using modern microscopy tools or by analyzing data gathered through laboratory trials. A model which can be used to monitor, observe and study the development of biofilm in an environment that mimics the oral cavity has been designed by Tang *et al.* (2003).

In the early days, the artificial mouth model was designed using glass cylindrical funnel (Pigman *et al.*, 1952; Pigman and Newbrun, 1962). This was later replaced by glass incubation chamber (Russell and Coulter, 1975). Subsequently, artificial mouth models called the Column System using pyrex glass tube (Sudo, 1977) and a culture vessel were designed (De Jong and Van Der Hoeven, 1987).

From 1990 onward, a complex artificial mouth model was developed. The model was called A Multi-Plaque Artificial Mouth, consisting of a glass cylinder containing five stations for plaque growth. This complex artificial mouth model can be used to monitor long-term growth of plaques (Sissons *et al.*, 1991, 1992; Shu *et al.*, 2000; Filoche *et al.*, 2004). An artificial mouth model reported

---

Corresponding Author: Dr. Zubaidah Hj Abd Rahim, Department of Oral Biology, Faculty of Dentistry,
University of Malaya, Kuala Lumpur 50603, Malaysia

by Busscher *et al.* (1992) was the Parallel-plate Flow Chamber. The model consists of glass-bottomed plate with connecting flasks.

Later, artificial mouth models using Constant Depth Film Fermentor (CDFF) were developed (Kinniment *et al.*, 1996; Pratten *et al.*, 1998; Pratten and Wilson, 1999; Pratten *et al.*, 2000; Hope and Wilson, 2003; Steinberg *et al.*, 2005; Lamfon *et al.*, 2005). The basis of the CDFF is a rotating turntable containing recessed cavities into which an appropriate substratum can be inserted. Some researchers subsequently used flow cells/flow chamber as artificial mouth models (Weiger *et al.*, 1999; Palmer *et al.*, 2001; Decker *et al.*, 2003; Foster and Kolenbrander, 2004). The flow cells/flow chamber usually consisted of glass slides, glass cover slips or enamel slides as substratum for biofilm formation. An artificial mouth model consisting of silicone rubber tubing packed with glass beads and connected to a peristaltic pump has been designed by Black *et al.* (2004).

In all of those models, various substrata that include hydroxyapatite beads, cover slips, glass beads and enamel are used for biofilm formation. Peristaltic pumps were used to supply the saliva and nutrients to the developing biofilm. There are attempts by some researchers (Filoche *et al.*, 2005; Takarada *et al.*, 2004; Park *et al.*, 2003; Fathilah and Rahim, 2003) to study biofilm formation by evaluating the bacterial adherence to glass surfaces under planktonic state. Microbes in oral biofilm however, may exhibit different properties from those in planktonic state (Black *et al.*, 2004; Ceri *et al.*, 1999).

The *in vivo* investigation of oral biofilm development is made difficult by its heterogeneity, limited access, variability and uncontrollable oral environments and ethical problems (Wong and Sissons, 2001). This triggers the interest to design an artificial mouth model to be referred as Nordini's Artificial Mouth (NAM) model for the development of oral biofilm. The reliability of the model was evaluated for use in the development of biofilm. Thus the main objective of this study was to determine the reproducibility of the bacterial populations formed in the biofilm between glass beads in one experiment and between different experiments. The efficiency of the model in maintaining conditions that mimics the oral environment was also validated.

MATERIALS AND METHODS

Glass Pasteur Pipette and Glass Beads Were Purchased from Merck, Germany

Bacterial reservoir and water bath were custom made and designed with dimensions suitable for the minimum space in the laboratory. Both were made from pyrex glass and consist of two parts (top and bottom) with a total height of 10 cm. The diameter of the water bath is 8.0 cm, whereas that of the bacterial reservoir, 6.5 cm. The top part of the water bath has a hole which can be used to fit in a thermometer while its bottom part has two opposite apertures. For the bacterial reservoir, the top part is provided with two openings which are opposite to one another.

Brain Heart Infusion agar and Brain Heart Infusion broth were purchased from Oxoid Ltd., Hampshire, England

Bacterial strains (*Strep. mutans* ATTC 25175) were purchased from ATTC, United States (US) in freeze-dried form.

Washing Procedures for the Apparatus and Glass Wares

All washables apparatus and glass wares were soaked overnight in a diluted solution of Decon 90. The glass wares were then sonicated in an Ultrasonicator for a few minutes followed by scrubbing with suitable brushes. The apparatus were rinsed thoroughly with tap water and re-rinsed with distilled water. The apparatus were then air-dried or oven-dried prior to sterilization.

Sterilisation and Aseptic Techniques

In this study, heat sterilization methods were employed. Moist heat-sterilisation was carried out by autoclaving at 121°C at 15 psi for 20 min. All microbiological media, solutions, glass wares, apparatus and other items that can withstand high temperature and pressure were sterilized using this method. Dry heat sterilization method, i.e., direct flaming was used to sterile the inoculating loops and hockey stick. All procedures, methods and practices that require aseptic techniques were carried out according to the method described by Benson (2002).

Setting up of the Nordini's Artificial Mouth (NAM) Model

A glass pasteur pipette was cut using a diamond blade to give a cylindrical glass chamber (1×6 cm) to represent the oral cavity. Six glass beads (3 mm in diameter) were placed along the length of the chamber and served as substrata for biofilm formation. The open-ends of the glass chamber were connected with rubber tubing to a bacterial reservoir via a peristaltic pump. The chamber was immersed in a water bath which functions as an incubator, with temperature fixed at 37°C to mimic the *in vivo* temperature of the mouth. A thermometer was fitted into the top part of the incubator to monitor the temperature of the system. This was to ensure that the biofilm develops in an environment that mimics that of the mouth. The peristaltic pump was used to maintain the flow rate that represents the flow rate of saliva in the oral cavity, of fluids coming from and fluids flowing back to the bacterial reservoir after passing through the NAM model.

Preparation of Sterile Saliva

Undiluted sterile saliva was prepared by a slight modification of the method described by De Jong and Van der Hoeven (1987). About 25 mL of Stimulated Whole Saliva (SWS) was collected everyday from a single volunteer. The volunteer was asked to chew on a sugar-free gum to stimulate saliva production. The collection of SWS was done using ice-chilled test tubes. The saliva was collected from a single volunteer to minimize any variations that may arise between individuals. The aggregation of protein in the SWS samples was minimized by adding 1,4-Dithio-D,L-threitol (DTT) to a concentration of 2.5 mM. Upon addition of DTT, the saliva was stirred slowly for 10 min before it was centrifuged at 864 x g for 30 min. The supernatant obtained was then filter-sterilized through a disposable 0.2 μm (Supor Membrane) low protein-binding filter (Acrodisc Syringe Filters, Pall Corp, USA) into sterile test tubes. The sterile SWS was then stored at -20°C. Prior to use, the SWS was thawed and centrifuged once again to remove any precipitate.

Preparation of Bacterial Suspension

The stock culture of *Strep. mutans* was thawed and then inoculated into 30 mL of sterilized BHI broth (1:100 v/v) accordingly to form the bacterial suspension. The concentration of the bacteria used in the study was adjusted using a spectrophotometer to give an absorbance of 0.144 at 550 nm wavelength. At this absorbance, the bacterial cell number is 10^8 cells mL^{-1} (Fathilah and Rahim, 2003). This procedure is important for the standardization of bacterial cell number in the suspension to be used in the study.

Preparation of Brain Heart Infusion (BHI) Broth Supplemented with 1% Sucrose

The preparation of BHI broth supplemented with 1% sucrose was similar to the preparation of BHI broth except for the addition of 1% sucrose to its total volume. The mixture was then sterilized by autoclaving and kept at 4°C until further use. This media was used as a reservoir for the bacteria.

Preparation of Brain Heart Infusion Agar (BHI Agar) Media

BHI agar plates were prepared according to the manufacturer's instruction (Oxoid Ltd.). The agar plates were stored in the fridge at 4°C until further use. These plates were used for the enumeration of the colony forming unit (CFU) of the cultured bacteria.

Experimental Procedure for the Development of Oral Biofilm

In the development of the biofilm, six glass beads were first coated with saliva by allowing the sterilized saliva to flow into the NAM model for two minutes at a rate of 0.3 mL min^{-1}. Following that, sterile distilled water was allowed to run into the system to rinse excess saliva from the glass beads. The glass beads covered by saliva represent the acquired pellicle. Bacterial inoculum (*Strep. mutans*) in the bacterial reservoir was then pumped into the system at a rate of 0.3 mL min^{-1} for 24 h to develop a 24 h-biofilm of *Strep. mutans*.

For blank control, bacteria-free inoculum was used. The experiment was repeated two times.

Analysis of the Oral Biofilm Formed in the NAM Model

The glass beads with the 24 h biofilm were analyzed for the number of bacterial cells attached to it. The glass beads located in the middle (M) and those nearest to the inlet (I) and outlet (O) tubing were chosen and analyzed for bacterial counts. The glass bead was individually placed in a test-tube containing Phosphate-Buffered Saline (PBS), sonicated for a few seconds and vortexed for one minute to dislodge the attached bacteria. The tube containing the dislodged bacteria was labeled as Tube 1 (T1) which contained undiluted bacterial count. Tube 1 was then diluted serially six times (1:10) using the phosphate buffer with the final dilution (6th dilutions) contained in Tube 7 (T7) (dilution factor $= 10^{-6}$). One hundred microliter of the bacterial suspensions from each of the tubes was drawn and plated on three separate BHI agar plates. These will give in total 21 BHI agar plates to be incubated aerobically at 37°C for 24 h. The counting of viable microorganisms was carried out on the following day. Plates from dilution which gave a cfu number of 30-300 cells were selected. The growth population cfu mL^{-1} were calculated using the formula:

$$\text{CFU count per plate} \times \text{dilution factor}$$

The result was expressed as mean of cfu count of three plates.

Validation of the Efficiency of the NAM Model in Maintaining the Preset Conditions

The NAM model was validated for its efficiency in maintaining temperature, flow rate and sterility throughout the experiment. For temperature, the water bath was preset at 37°C to mimic the human oral cavity temperature. It was constantly monitored by the thermometer and recorded every 2 h for 6 h and this was repeated for three different days. For flow rate, it was preset at 0.3 mL min^{-1} to represent the flow rate of saliva in the oral cavity under unstimulated condition (Edgar and O'Mullane, 2004). It was monitored and recorded every 2 h for 6 h and repeated for three different days. For sterility, the sterility of the apparatus was determined in three different days.

Statistical Analysis

Analysis of variance (ANOVA) was used in the statistical analysis. A p-value of less than 0.05 is considered significant. Comparisons of the bacterial counts were carried out between the beads within an experiment and between experiments.

RESULTS AND DISCUSSION

Figure 1 represents the NAM model used in the study. It was found that the bacterial population (cfu mL^{-1}) in the biofilm formed on the glass beads (M, I and O) in each experiment were not

Table 1: The bacterial population present in the biofilm formed on each bead was determined and analyzed statistically using ANOVA

Bead	Experiment 1 Bacterial population (10^3)	Experiment 2 Bacterial population (10^3)	p-value
(I) Nearest to the inlet	129.3 ± 20.3	130.7 ± 20.3	$p > 0.6$
(M) Middle	123.3 ± 16.3	122.7 ± 23.3	$p > 0.6$
(O) Nearest to the outlet	119.3 ± 22.3	134.3 ± 36.0	$p > 0.6$

The bacterial population is expressed as the mean ± standard deviation of three determinations

Table 2: Comparison of the initial and final readings of the parameters used in the study

Parameters	Initial readings	Final readings	Deviations (%)
Temperature	37°C	36.7 ± 0.5	0.8
Flow rate	0.3 (mL min^{-1})	0.3 ± 0.0 (mL min^{-1})	None
Sterility	No bacterial growth	No bacterial growth	None

Fig. 1: NAM model (A) inside the water bath (incubator) (B) connected to a reservoir (C) via a peristaltic pump. Inset is the enlarged picture of A (indicated by the arrow)

statistically different (p>0.6) from one another. The standard deviations between experiments 1 and 2 were found to be within 4-5 % (Table 1). The sterility of and temperature and flow rate set for the NAM model prior to the experiment was found to be maintained throughout the experiment (Table 2).

Attempts to simulate and study oral biofilms formation *in vitro* are not new. Since 1952, many researchers (Pigman *et al.*, 1952; Pigman and Newburn, 1962; Russell and Coulter, 1975; Sudo, 1977; De Jong and Van Der Hoeven, 1987; Busscher *et al.*, 1992; Kinniment *et al.*, 1996; Pratten *et al.*, 1998; Pratten and Wilson, 1999; Weiger *et al.*, 1999; Pratten *et al.*, 2000; Palmer *et al.*, 2001; Hope and Wilson, 2003; Decker *et al.*, 2003; Black *et al.*, 2004; Foster and Kolenbrander, 2004; Lamfon *et al.*, 2005; Steinberg *et al.*, 2005) have already conceived such experiments. Many of the so-called artificial mouth models have used a single chamber containing an enamel surface upon which nutrient or saliva was allowed to flow or drip. The chamber containing the enamel is inoculated directly with oral bacteria

and the supply of nutrient allows for colonization of the surface and development of an experimental biofilm. All of these models were designed with the recognition that the flow of saliva and nutrient over the surface are important factors for the development of an oral biofilm.

In this study, the NAM model used a glass chamber to mimic the oral cavity and the glass beads to serve as substrata for biofilm development. The temperature was fixed at 37°C to mimic the *in vivo* temperature of the mouth and maintained throughout the experiment by immersing the chamber in a water bath which functions as an incubator. A thermometer placed in the incubator kept the temperature of the system in check. This was to ensure that the biofilm develops throughout the experiment in an environment that mimics that of the mouth. Before the bacterial inoculum was pumped into the system, the beads were first coated with saliva to allow for a layer of acquired pellicle or experimental pellicle to form. The flow rate of fluids going in and leaving the chamber was controlled by the peristaltic pump.

The NAM model differs from the previously published models in three aspects. Firstly, it employs a continuous culture system (bacterial reservoir). The bacterial reservoir which contains a population of oral microorganisms in a broth-containing medium supplemented with sucrose is supplied continuously to the biofilm-forming surfaces (glass beads). The top and bottom part of the water bath and reservoir are kept tight with screw-clamps. This ensures that the environment of the bacterial reservoir and the water bath is sterile throughout the experiment. Secondly, it allows for variability of the environment of the bacterial reservoir. Extra nutrient can be added in order to maximize the formation of oral biofilms without affecting the influx of bacteria into the system. The environment can also be varied to suit the requirement for the study. Thirdly, it requires a small space in the laboratory (60 cm (long) × 43 cm (wide)).

The NAM model is valid for use in the development of biofilm as the results obtained on the study are reproducible. This is indicated by the bacterial population (cfu mL^{-1}) of the biofilm formed on the glass beads (M, O and I) which was not significantly different to one another (p>0.6). The standard deviations between the two experiments are within 4-5% implying its usage with good reproducibility.

CONCLUSION

The NAM model can be used in the development of oral biofilms with reproducibility and reliability.

ACKNOWLEDGMENT

REFERENCES

Benson, H.J., 2002. Microbiological Applications: Laboratory Manual in General Microbiology. 8th Edn. McGraw-Hill Higher Education. Boston.

Black, C., I. Allen, S.K. Ford, M. Wilson and R. McNab, 2004. Biofilm-specific surface properties and protein expression in oral *streptococcus sanguis*. Arch. Oral. Biol., 49 (4): 295-304.

Busscher, H.J., G.I. Doornbusch and H.C. Van Der Mei, 1992. Adhesion of mutans streptococci to glass with and without a salivary coating as studied in a parallel-plate flow chambers. J. Dent. Res., 71 (3): 491-500.

Ceri, H., M.E. Olson, C. Stremick, R.R. Read, D. Morck and A. Buret, 1999. The calgary biofilm Device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J. Clin. Microbiol., 37 (6): 1771-1776.

Decker, E.M., R. Weige, C. Von, Ohle, I. Wiech and M. Brecx, 2003. Comparison of antiadhesive and antibacterial effects of antiseptics on *Streptococcus sanguinis*. Clin. Oral. Invest., 7 (2): 98-102.

De Jong, M.H. and J.S. Van Der Hoeven, 1987. The growth of oral bacteria on saliva. J. Dent. Res., 66 (2): 498-505.

Edgar, W.M. and D.M. O'Mullane, 2004. Saliva and Oral Health. 3rd Edn. British Dental Association. London.

Fathilah, A.R. and Z.H.A. Rahim, 2003. The anti-adherence effect of *Piper betle* and *P.sidium guajava* extracts on the adhesion of early colonizers in dental plaque to saliva-coated glass surfaces. J. Oral. Sci., 45 (4): 201-206.

Filoche, S.K., S.A. Anderson and C.H. Sissons, 2004. Biofilm growth of *Lactobacillus* sp. is promoted by *Actinomyces* sp. and *Streptococcus mutans*. Oral. Microbiol. Immunol., 19 (5): 322-326.

Filoche, S.K., K. Soma and C.H. Sissons, 2005. Antimicrobial effects of essential oils in combination with chlorhexidine digluconate. Oral. Microbiol. Immunol., 20 (4): 221-225.

Foster, J.S. and P.E. Kolenbrander, 2004. Development of a multispecies oral bacterial community in a saliva-conditioned flow cell. Applied Environ. Microbiol., 70 (7): 4340-4348.

Hope, C.K. and M.C. Wilson, 2003. Measuring the thickness of an outer layer of viable bacteria in an oral biofilm by viability mapping. J. Microbiol. Methods, 54 (3): 403-410.

Kinniment, S.L., J.W.T. Wimpenny, D. Adams and P.D. Marsh, 1996. Development of a steady-state oral microbial community using the constant-depth film fermenter. Microbiology, 142 (Pt 3): 631-638.

Lamfon, H., Z. Al-Karaawi, M. McCullough, S.R. Porter and J. Pratten, 2005. Composition of *in vitro* denture plaque biofilms and susceptibility to antifungals. FEMS. Microbiol. Lett., 242 (2): 345-351.

Palmer, R.J. Jr., K. Kazmerzak, M.C. Hansen and P.E. Kolenbrander, 2001. Mutualism vs Independence: Strategies of mixed-species oral biofilms *in vitro* using saliva as the sole nutrient source. Infect. Immun., 69 (9): 5794-5804.

Park, K.M., J.S. You, H.Y. Lee, N.I. Baek and J.K. Hwang, 2003. Kuwanon G: An antibacterial agent from the root bark of *Morus alba* against oral pathogens. J. Ethnopharm., 84 (2-3): 181-185.

Pigman, W., H.C. Jr. Elliott and R.O. Laffre, 1952. An artificial mouth for caries research. J. Dent. Res., 31 (5): 627-633.

Pigman, W. and E. Newbrun, 1962. Evaluation of anticaries agents by the use of the artificial mouth. J. Dent. Res., 41: 1304-1311.

Pratten, J., A.W. Smith and M. Wilson, 1998. Response of single species biofilms and microcosm dental plaques to pulsing with chlorhexidine. J. Antimicrob. Chemother., 42 (4): 453-459.

Pratten, J. and M. Wilson, 1999. Antimicrobial susceptibility and composition of microcosm dental plaques supplemented with sucrose. Antimicrob. Agents Chemother., 43 (7): 1595-1599.

Pratten, J., C.S. Andrews, D.Q.M. Craig and M. Wilson, 2000. Structural studies of microcosm dental plaques grown under nutritional conditions. FEMS. Microbiol. Lett., 189 (2): 215-218.

Russell, C. and W.A. Coulter, 1975. Continous monitoring of pH and Eh in bacterial plaque growth on a tooth in an artificial mouth. Applied Microbiol., 29 (2): 141-144.

Shu, M., L. Wong, J.H. Miller and C.H. Sissons, 2000. Development of multispecies consortia biofilms of oral bacteria as an enamel and root caries model system. Arch. Oral. Biol., 45 (1): 27-40.

Sissons, C.H., T.W. Cutress, M.P. Hofffman and J.St.J. Wakefield, 1991. A multistation dental plaque microcosm (artificial mouth) for the study of plaque growth, metabolism, pH and mineralization. J. Dent. Res., 70 (11): 1409-1416.

Sissons, C.H., T.W. Cutress, G. Faulds and L. Wong, 1992. pH responses to sucrose and the formation of pH gradients in thick artificial mouth microcosm plaques. Arch. Oral. Biol., 37 (11): 913-922.

Steinberg, D., M. Feldman, I. Ofek and E.I. Weiss, 2005. Cranberry high molecular weight constituents promote *Streptococcus sobrinus* desorption from artificial biofilm. Int. J. Antimicrob. Agents, 25 (3): 247-251.

Sudo, S.Z., 1977. Continous culture of mixed oral flora on hydroxyapatite-coated glass beads. Applied Environ. Microbiol., 33 (2): 450-458.

Takarada, K., R. Kimizuka, N. Takahashi, K. Honma, K. Okuda and T. Kato, 2004. A comparison of the antibacterial efficacies of essential oils against oral pathogens. Oral. Microbiol. Immunol., 19 (1): 61-64.

Tang G., H.K. Yip, T.W. Cutress and L.P. Samaranayake, 2003. Artificial mouth model systems and their contribution to caries research: A review. J. Dent., 31 (3): 161-171.

Weiger, R., E.M. Decker, G. Krastl and M. Brecx, 1999. Deposition and retention of vital and dead *Streptococcus sanguinis* cells on glass surfaces in a flow-chamber system. Arch. Oral. Biol., 44 (8): 621-628.

Wong, L. and C.H. Sissons, 2001. A comparison of human dental plaque microcosm biofilms grown in an undefined medium and a chemically defined artificial saliva. Arch. Oral. Biol., 46 (6): 477-486.