CHAPTER III

MATERIALS AND METHODS

1. Place

1.1 All of the population in this study was collected from the Out-Patient-Department of Oral Medicine, Faculty of Dentistry, Chulalongkorn University.

1.2 Culturing, measuring, and identification of yeasts were done at the Mycology Laboratory, Department of Microbiology, Faculty of Medicine, and Department of Microbiology, Faculty of Dentistry, Chulalongkorn University.

2. Population and Sampling Method

2.1 Control I : normal healthy oral mucosa (Group 1)

Seventy-three of the fifth-year and sixth-year dental students at Chulalongkorn University represented normal control group with good general health and oral hygiene and without any dental appliance. Individuals with a medical condition or taking drugs likely to influence, such as antibiotics, steroids, and immunosuppressive drugs, were excluded.

2.2 Control II : normal healthy palatal mucosa with a dental appliance (Group 2)

There were 27 healthy and good oral hygiene patients with a dental appliance, such as orthodontic plate, temporary plate, partial denture, and full denture, who attended the Out-Patient-Department of Oral Medicine, Faculty of Dentistry, Chulalongkorn University, during May 1988 - February 1989. They were not taking any kinds of antibiotics, steroids, or immunosuppressive drugs and had no history of oral candidiasis.

2.3 Denture stomatitis (denture sore mouth, or DSM, Group 3)

There were 40 denture stomatitis patients with generalized diffuse inflammation of the entire or almost entire denture palatal mucosa who attended the Out-Patient-Department of Oral Medicine, Faculty of Dentistry, Chulalongkorn University, during May 1988 - February 1989. The dental appliances were temporary plate, partial denture, and full denture. Individuals with predisposing factors, such as endocrine disorders, blood dyscrasis, and patients who recieved long-term antibiotics therapy, corticosteroids, or immunosuppressive drugs, or suffered from nutritional deficiency, cancer, or defects in cell-mediated immunity were excluded.

3. Method of Examination

To standardize the effect of diurnal variation, meals and toothbrushing, investigations were carried out at the same time of day (midmorning), at least two hours after eating, drinking, or any oral hygiene procedure. The methods of examination used were as follows:

3.1 Questionaire

The age, sex, dental and medical history, and drug therapy were recorded.

3.2 Oral examination

The appearance of the oral mucosa was noted. Whenever practical, the salivary sample and imprint culture techniques for detecting candida carriers were compared, both in the healthy subjects (Control I and Control II) and the oral candidiasis group.

4. Microbiological Methods

4.1 Materials

4.1.1 Examination set : exploror, mouth mirror, forceps

4.1.2 Sabouraud Dextrose Broth (Difco)

4.1.3 Sabouraud Dextrose Agar (Difco)

4.1.4 Sabouraud Dextrose Agar (Difco) + Sodium benzyl penicillin (Pegemex, Dumex) + Streptomycin sulfate (Streptomycin, Dumex)

4.1.5 Petri dish of sterile squares of plastic foam pads (2.5 x 2.5 cm)

4.1.6 Alcohol lamp

4.1.7 Sterile 20 ml glass container

4.2 Salivary samples

Each participant spat approximately 2 ml of mixed whole unstimulated saliva into a sterile glass container which was vibrated on a bench vibrator for 30 seconds to disaggregate the organisms. The density of candidal organism in saliva was determined [Williamson (40)]. The 0.2 ml of saliva was triplicately plated on Sabouraud Agar into which 1.5 mg/ml sodium benzyl penicillin and 5 mg/ml streptomycin sulfate had been incorporated to suppress bacterial growth. A surface candidal colony count was performed after 48 hours of incubation at 37 °C.

4.3 Imprint culture [Arendorf and Walker (34)]

Sterile squares of plastic foam pads (2.5 x 2.5 cm) were dipped in Sabouraud Broth and pressed for 60 seconds on the various oral mucosal or denture surfaces, positioned in oral sites as follows :

Anterior palate (AP)	The anterior edge of the pad was placed on an imaginary line connecting the mesial surfaces of the canines.

Posterior palate (PP)	The posterior edge of the foam pad was placed on an imaginary line connecting the distal surfaces of the maxillary second molars.
Right and left buccal mucosa (RB, LB)	The anterior edge of the foam pad was placed in line with the distal surfaces of the mandibular first premolar.
Right and left commissures (RC, LC)	Opposite corners of the pad were positioned on the midline of the upper and lower lips in the commissural areas so that half of the surface area sampled was on the mucosa and half on the skin.
Anterior dorsum ofthe tongue (AT)	The corners of the anterior edge of the pad were positioned so that they just touched the lateral margins of the protruded tongue.
Posterior dorsum of the tongue (PT)	The pad was positioned in the middle of the protruded tongue with the posterior edge placed on an imaginary line connecting the distal surfaces of the mandibular second molars.

Mandibular anterior labial sulcus (LA)	The foam pad was placed in the midline of the sulcus with the posterior edge aligned just below the free gingival margin of the mandibular incisors.
Floor of mouth (F1)	The pad was placed in the midline of the floor of the mouth, beneath the tongue, with the anterior edge aligned just below the free gingival margin of the mandibular incisors.
Fitting surface of denture (D)	The pad was placed in the central fitting surface of denture.

The foam pad was then pressed firmly on a Sabouraud Agar plate and left in place for the first 8 out of 48 hours of incubation at 37 °C. Pilot studies showed that a 2.5 x 2.5 cm square of plastic foam was the largest which could be placed on the chosen sites without contamination from adjacent oral structures. The candidal density at each site was determined by measuring the area of the cultural growth on agar media. Providing the growth into 5 levels as follows:

0	=	No growth of yeasts
1	=	Yeasts grew from 1 colony up to 25% area of foam pad on agar
2	=	Yeasts grew in more than 25% up to 50% area of foam pad on agar
3	=	Yeasts grew in more than 50% up to 75% area of foam pad on agar
4	=	Yeasts grew in more than 75% area of foam pad on agar

After the growth of yeasts from various sites of the oral mucosa and denture base, a sample of yeast from each area was collected. If there were other different colour and texture of yeast colonies, more than one colony from that area would have been collected. To inoculate the sample in broth culture, it was incubated at 37 ^{o}C for 24 hours, then streaked on Sabouraud Agar plate to get pure isolated colonies from different areas, and finally identified.

4.4 Identification methods

Presumptive identification of each single isolated colony was made by using the procedures described by McGinis (41) and Beneke (42), which can be summarized into Table 1 and Chart 1. Final identification of each isolate was made by using modified criteria of

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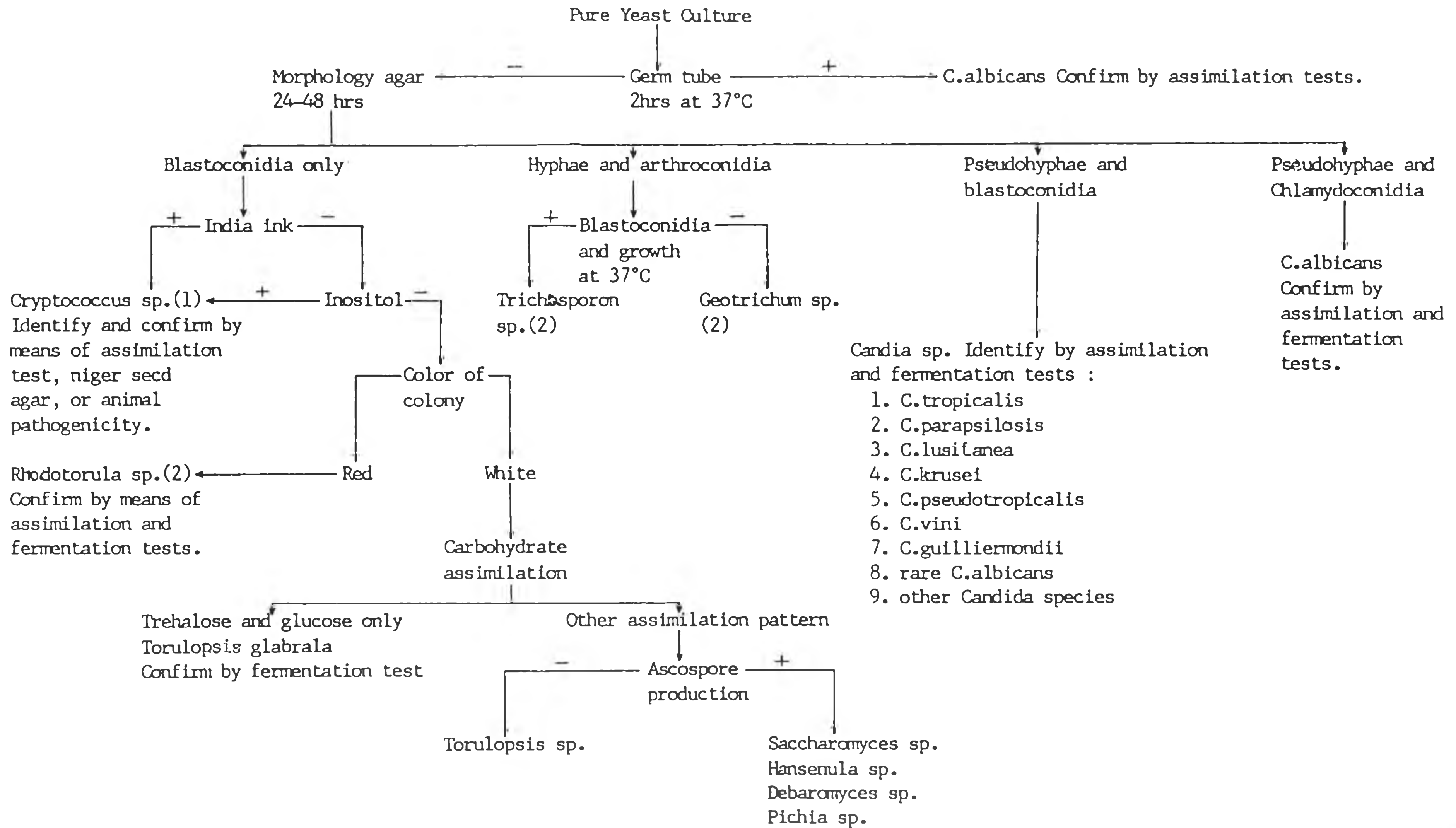

Chart 1 An approach to the identification of yeast isolants.

Table 1. Physiologic Characteristics of *Candida* spp. and Other Yeasts

Organism	Surface Growth on Sabouraud Broth	Sugar Assimilation												Sugar Fermentation					
		Glucose	Maltose	Sucrose	Lactose	Galactose	Melibiose	Cellobiose	Inositol	Xylose	Raffinose	Trehalose	Dulcitol	Glucose	Maltose	Sucrose	Lactose	Galactose	Trehalose
C. albicans	0	+	+	+	0	+	0	0	0	+	0	+	0	AG	AG	A	0	AG or A	AG or A
C. stellatoidea	0	+	+	0	0	+	0	0	0	+	0	+*	0	AG	AG	0	0	0	0
C. tropicalis	+ (film)	+	+	+	0	+	0	+*	0	+	0	+	0	AG	AG	AG	0	AG	AG
C. pseudo-tropicalis	0	+	0	+	+	+	0	+	0	+*	+	0	0	AG	0	AG	AG	AG	AG
C. krusei	+ (film)	+	0	0	0	0	0	0	0	0	0	0	0	AG	0	0	0	0	0
C. parapsilosis	0	+	+	+	0	+	0	0	0	+	0	+	0	AG	0	0	0	AG	AG or A
C. guilliermondii	0	+	+	+	0	+	+	+	0	+	+	+	+	AG	0	AG	0	AG or A	AG or A
C. rugosa	0	+	0	0	0	0	+	0	0	+	0	0	0	0*	0	0	0	0	0
Saccharomyces cerevisiae	0	+	+	+	0	+	0	0	0	0	+	+*	0	AG	AG	AG	0	AG	AG*
Geotrichum candidum	0	+	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0
Torulopsis glabrata	0	+	0	0	0	0	0	0	0	0	0	+	0	AG	0	0	0	0	+
Trichosporon cutaneum	0	+	+*	+*	+	+	+*	+	+*	+	+*	+++*	+*	0 or A	0 or A	0 or A	0 or A	0 or A	0 or A

* Strain variation.
A = acid; G = gas; + = reaction; 0 = no reaction.

Kreger-van Rij (43). The identification of *Candida* could be performed by using either chlamydoconidia formation, carbohydrate fermentation test, carbohydrate assimilation test, urease test or nitrate assimilation test.

4.4.1 Chlamydoconidia formation (44)

Materials

1) Glutinous rice Tween 80 agar plate (see Appendix)
2) Sterile cover slip
3) Tested strain

Method

(1) Glutinous rice Tween 80 agar plate was divided into 4 sections.
(2) The active growth yeast was streaked in a quarter of the divided plate.
(3) Sterile cover slip was placed on the streak.
(4) The inoculated plate was incubated in 25 °C incubator for 24 hours and examined for chlamydoconidia production under high air-dry objective lens.

4.4.2 Carbohydrate fermentation test

Materials

1) Six tubes of fermentation broth (see Appendix) with Durham tube, each containing one of the following carbohydrates: glucose, sucrose, maltose, trehalose, galactose, and lactose.
2) Sterile liquid paraffin
3) Sterile distilled water
4) Tested strain

Method

(1) The active growth yeast was suspended in sterile distilled water about no. 4 - 5 McFarland density.
(2) One milliliter of yeast suspension was pipetted into tube of six different carbohydrate fermentation broths.
(3) Sterile liquid paraffin, about 1.0 - 1.5 cm thickness, is overlayed upon the inoculated broth.

(4) The tubes were incubated at room temperature and examined for the production of gas in Durham tube for 3, 5, 7, and 14 days.

4.4.3 Carbohydrate assimilation test (Auxanographic method)

Materials

1) Six tubes of yeast nitrogen base (YNB) agar with indicator
2) Three sterile plates, 150 mm
3) Fifteen different kinds of carbohydrate discs (see Appendix)
4) Sterile distilled water
5) Forceps
6) Tested strain

Mothod

(1) YNB agar was melted and allowed to cool to 50 ^{o}C in water bath.
(2) The active growth yeast was suspended in sterile water about no. 4 - 5 McFarland density.

(3) Three milliliters of yeast suspension was pipetted in 6 tubes of melted YNB agar and mixed thoroughly.

(4) Two tubes of inoculated YNB agar were poured in a plate and allowed to harden.

(5) Fifteen different carbohydrate discs were placed on the surface of the hardened agar as shown in Fig. 1.

(6) Plates were incubated at room temperature for 24-72 hours.

(7) Positive results should be observed by changing of color from violet to yellow and/or the growth of yeast colonies around each of the discs of carbohydrates at 24, 48, and 72 hours.

4.4.4 Nitrate assimilation test (Auxanographic method)

Materials

1) Yeast carbon base agar (see Appendix)

2) Potassium nitrate disc and peptone disc (see Appendix)
3) Sterile plates
4) Tested strain

Method

(1) The active growth yeast was suspended in sterile distilled water about 0.5 McFarland density.
(2) Half milliliter of yeast suspension was pipetted into a melted (50 °C) yeast carbon base agar tube, mixed thoroughly, and poured into a plate, the agar was allowed to harden.
(3) Plate was divided into two parts, potassium nitrate disc was placed on one side, and peptone disc on the other as the control of positive growth.
(4) Plate was incubated at room temperature for 3 days and examined for the growing zone around each disc indicated by a positive result or assimilated.

4.4.5 Urease test

Materials

1) Christensen urea agar slant (see Appendix)
2) Tested strain

Method

(1) The active growth yeast was streaked on Christensen urea agar slant.
(2) The inoculated slant was incubated at room temperature for 3 days and was examined for the change of color from orange to brightly pink as the positive test.

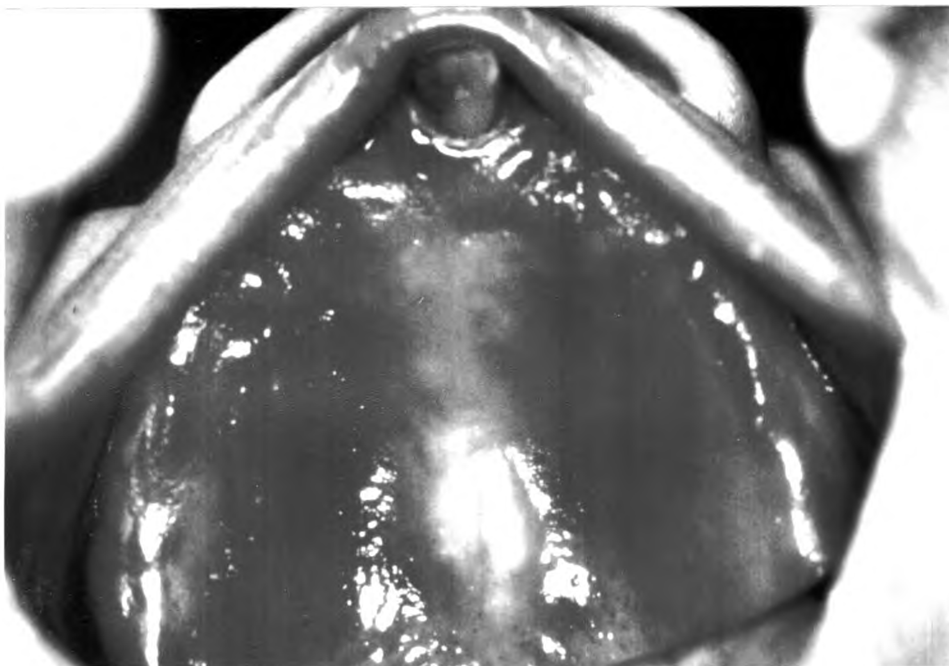

Figure 1 Denture stomatitis

Figure 2 Complete denture (full denture) and temporary plate

Figure 3 Material and media for imprint culture

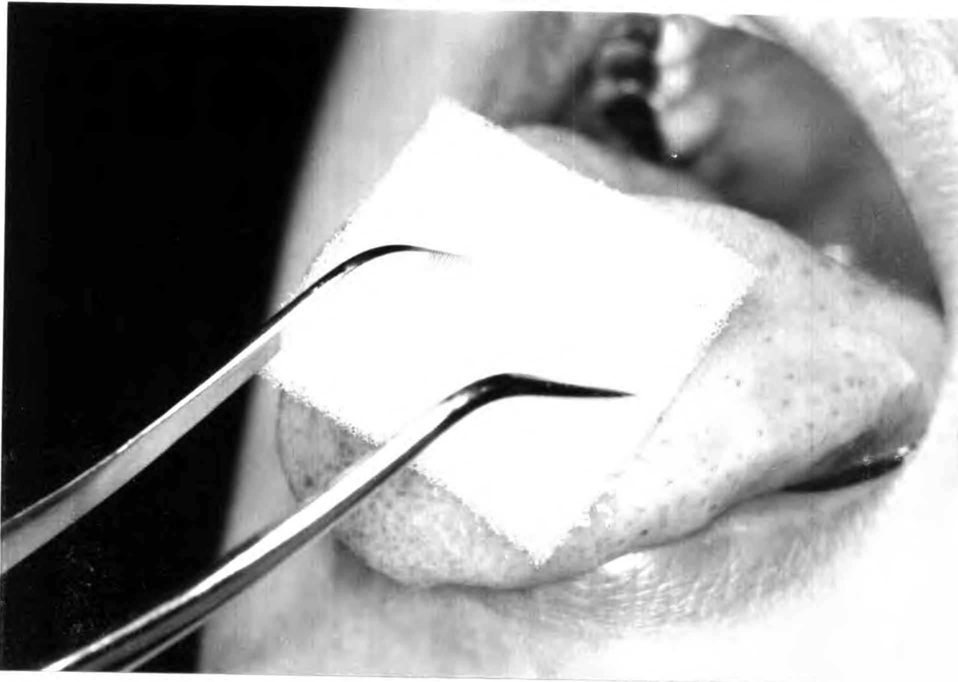

Figure 4 Imprint culture of anterior dorsum of the tongue (AT)

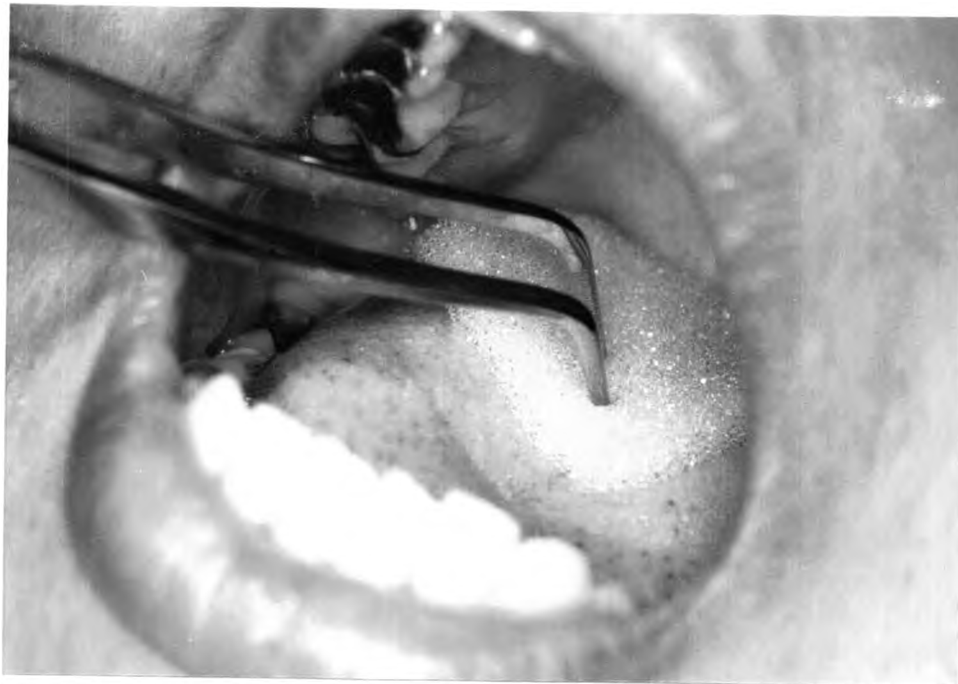

Figure 5 Imprint culture of posterior dorsum of the tongue (PT)

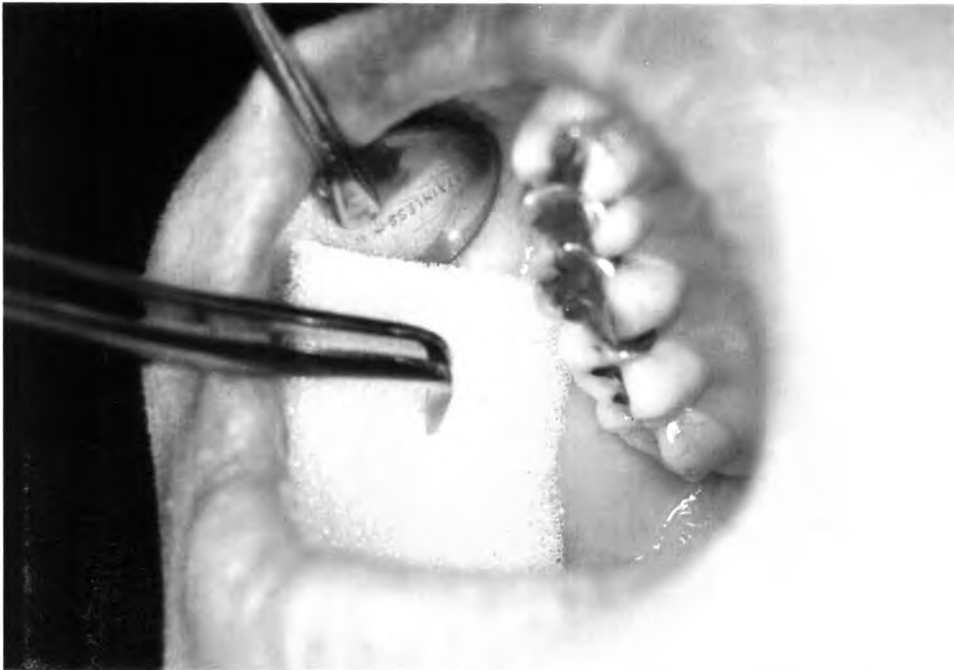

Figure 6 Imprint culture of right and left buccal mucosa (RB, LB)

Figure 7 Imprint culture of right and left commissure (RC, LC)

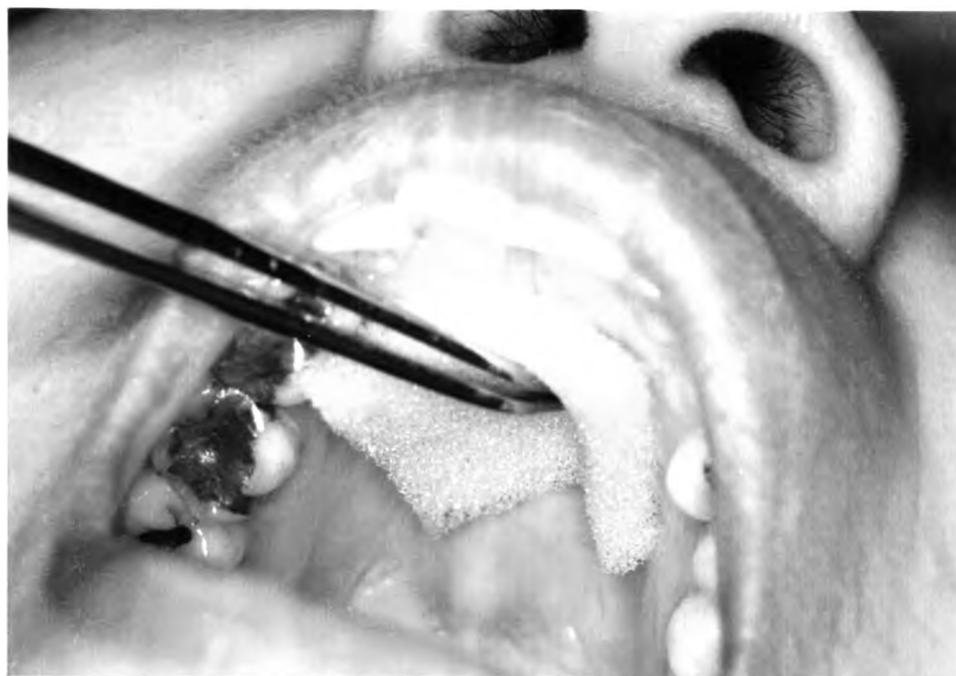

Figure 8 Imprint culture of anterior palate (AP)

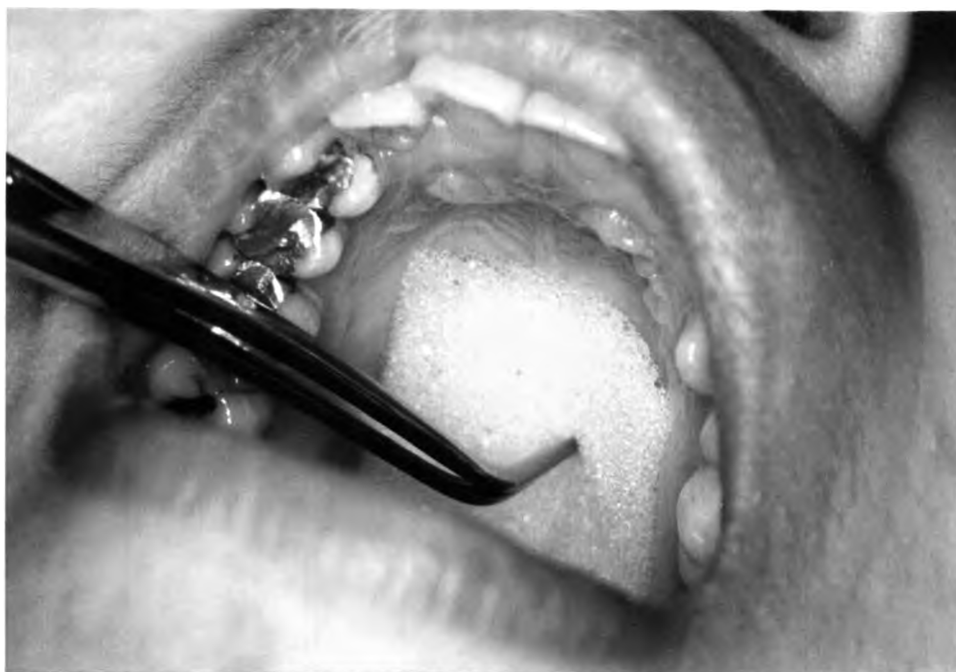

Figure 9 Imprint culture of posterior palate (PP)

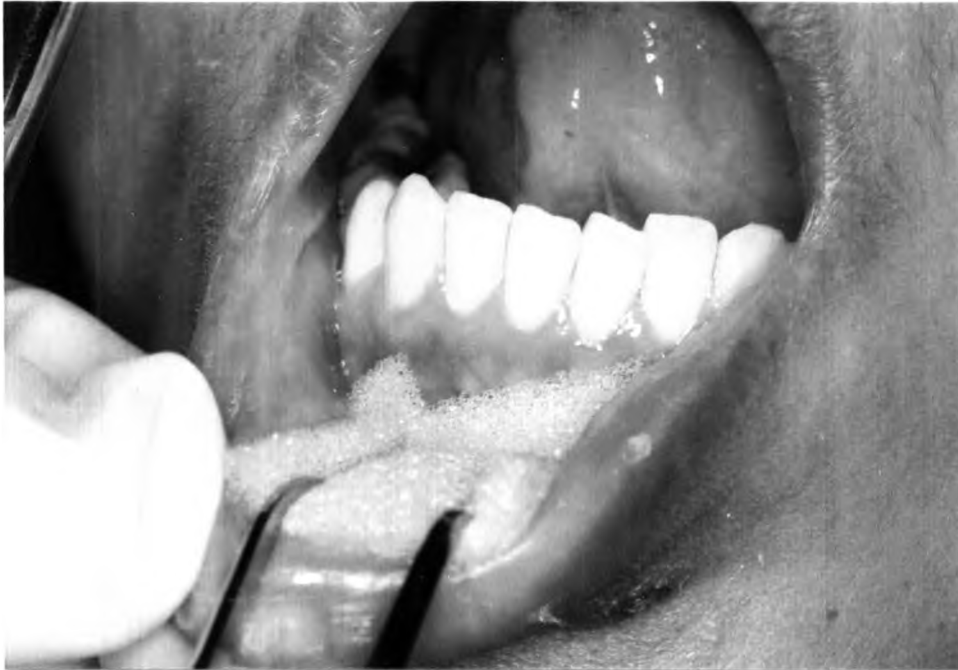

Figure 10 Imprint culture of mandibular anterior labial mucosa (LA)

Figure 11 Imprint culture of floor of mouth (FI)

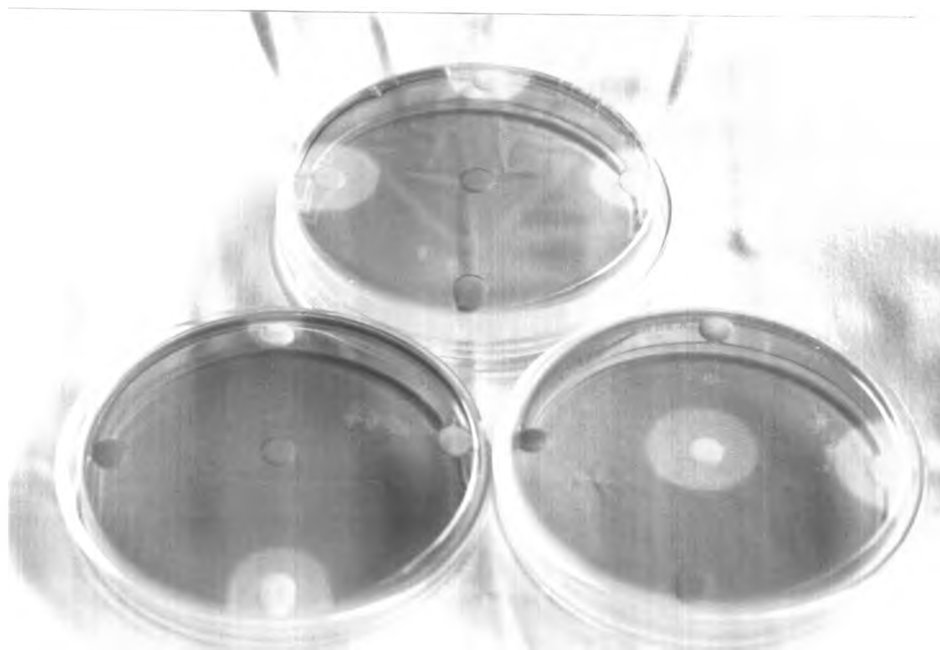

Figure 12 Demonstration of carbohydrate disc in the assimilation for each isolated plate.

Plate I : (upper) Glucose, maltose, sucrose, lactose, sorbose.

Plate II : (lower left) Galactose, cellobiose, melibiose, raffinose, soluble starch.

Plate III : (lower right) Xylose, arabinose, inositol, dulcitol, trehalose.

Analysis of Data

Mean, $\bar{X}$ = $(\sum X)/n$

Standard deviation, SD = $\sqrt{\sum (X - \bar{X})^2/n}$

Chi-square Test, χ^2 = $\sum_{i=1}^{k} (O_i - E_i)^2/E_i$

Analysis of Variance:

The total sum of squares is defined as

$$S.S. = \sum_{i=1}^{n} \sum_{j=1}^{k} (X_{ij} - \bar{X})^2$$

The among-means sum of squares is defined as

$$S.S.T. = n \sum_{j=1}^{k} (\bar{X}_j - \bar{X})^2$$

The within-means sum of squares is defined as

$$S.S.E. = \sum_{i=1}^{n} \sum_{j=1}^{k} (X_{ij} - \bar{X}_j)^2$$

Degree of freedom = df

Variance Ratio = VR

Anova Table Treatment on Group

Source of Variation	Sum of Squares	df	Mean Squares	VR	$VR_{0.95}$
Total	$S.S. = \sum_{i=1}^{n} \sum_{j=1}^{k} X_{ij}^2 - C$	kn-1			
Between mean	$S.S.T. = (\frac{1}{n}) \sum_{j=1}^{k} T_j^2 - C$	k-1	$S_t^2 = \frac{S.S.T.}{k-1}$	$\frac{S_t^2}{S_p^2}$	Tabular Value
Within mean	$S.S.E. = S.S. - S.S.T.$	k(n-1)	$S_p^2 = \frac{S.S.E.}{k(n-1)}$		

Duncan's New Multiple Range Test for Group

Significant Studentized Range (SSR) from Table

	p	2	3	4
SSR	df			

Least Significant Range

$$LSR = S\,(SSR)\sqrt{\frac{1}{2}\left(\frac{1}{n_i} - \frac{1}{n_j}\right)}$$

Error variance $S_{\bar{X}} = \sqrt{S_p^2/n}$

PAIRS	$S_{\bar{X}}$	$T_i - T_j$	LSR