Growth and Amine Production by Anaerobic Bacteria in the Human Vagina

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Introduction

Very little is known about the ecology of anaerobic bacteria in the human vagina. Normally a lactobacillus- dominated environement, the vaginal tract usually has a pH of 4.5 (Rein, 1995). At this acidity there are few bacteria present which are not lactic acid producers. It is now known that the mature vagina is colonized by a variety if microbes, primarily obligate and facultative anaerobes. These include Lactobacillus acidophilus, Viridans streptococci, and Staphylococcus epidermis (Rein, 1995). Most population variation is attributed to hormonal fluctuations. Our descriptive knowledge of vaginal microbiology is primitive when it involves what other factors influence diversity.

Bacterial vaginosis (BV) is an infectious disease affecting the female reproductive system that is believed to be caused by a consortium of these anaerobic bacteria. Symptoms of BV include vaginal itching and excessive discharge; usually yellow-green in color and considerably malodorous. The offensive odor is thought to caused by the production of cadaverine, putrescine, and trimethylamine. It is not known what triggers the change from a normal biota to the mixed, predominantly anaerobic community linked to vaginosis which includes Mobiluncus. No research to date has definitively described this heterogeneous mix of anaerobes.

The purpose of this research is to enrich for this poorly studied group of urogenital tract microorganisms. Because Mobiluncus is thought to co-occur with many other anaerobes, the approach if this study will be to optimize conditions for its growth. Other microbes which grow will be characterized along with the target organism. The investigation is aimed at culturing and characterizing Mobiluncus and other pathogenic anaerobes present in the vagina. Normal vaginal samples will be cultured using a variety of carbohydrate-enriched media. Cells will be identified through phase contrast microscopy, gram stain, catalase tests, and oxidase tests. Metabolic products will be detected using HPLC methodology.

Materials and Methods

Sample Preparation

Vaginal smears were taken from two women (AS, DLH) complaining of no illness or symptoms. Smears were suspended in 5ml of composite media (described in media preparation). Two inoculating suspensions were made from each: one fresh and one cold treated. Fresh samples were simply resuspended and used for innoculation. Cold enriched samples were incubated at 20C for 45 minutes, then for 12 hours at 4C. This technique was employed because it has been demonstrated as encouraging the growth of Mobiluncus species (Smith & Moore, 1988).

Media Preparation

Four types of media were used in an effort to isolate as many different microorganisms as possible: 1% glycogen supplemented brain heart infusion agar (G), 1% glycogen supplemented brain heart infusion agar enriched with hemin (G+), .05% mucin supplemented brain heart infusion agar enriched with hemin (M), and sheep's blood brain heart infusion agar (**B,** available form BBL). Glycogen and mucin were provided as carbohydrate sources. Roll tubes were prepared using G, G+, and M agar for anaerobic cultures (N_2 w/ 10%CO_2 atmosphere). Enriched agars were prepared with hemin because many enteric microorganisms need an iron rich blood based medium. Sheep's blood BHI is a commercially available plate agar which has been successfully used for culturing Mobiluncus. (Smith and Moore, 1988; Hammann et. al., 1983). Cultures grown on brain heart infusion agars were later streaked onto blood plates and vice versa for comparative analysis.

A composite 1% glycogen media (.0005g hemin, 5g glygogen, .000375g vancomycin, .05 neomycin in 500ml distilled H_20) was prepared for use during sample collection and for liquid

culture. Preexisting anaerobic basal media was often used for resuspension of cells when the composite media was too nutrient rich and would interfere with an assay.

Identification of Bacteria

To elucidate characteristics of cultured cells, catalase, oxidase, gram stain, and hemolytic tests were performed. Gram stain and oxidase tests are commercially available (Difco). Hemolysis was assessed by observing isolates grown on sheep blood agar plates (BBL). Hemolytic organisms will show a golden (beta) or greenish (alpha) discoloration of blood agar. Hydrogen Peroxide (3%) was used for catalase assay.

For in situ hybridization, mature cells from 5 different plates and 3 used. Samples were suspended in anaerobic basal media and chilled on ice for 5 minutes. The sample (1 ml) was centrifuged at top speed for 5 minutes. The pellet was resuspended and washed twice in iced PBS, centrifuging after each wash. Afterwards, the final pellet was resuspended 1:100 in cold PBS and applied to subbed slides in 15 microliter aliquots. Subbed slides were welled slides which had been washed 20 times in H_2O, twice in dH_2O, baked 4-8 hours at 200C, and then dipped in subbing soltuion (0.1% gelatin and 01% $CrK(SO_4)$ $12H_2O$). They were dried in a 37C incubator. Before adding the probes, culture slides were treated with enthanol/formaldehyde solution (90:10) for 5 minutes at room temperature. To make sure all fixative was removed, slides were rinsed twice in dH_2O and air dried. Low G+C, high G+C, and flavobacteria probes (40microliters) were applied to each well and incubated overnight. Low G+C and flavobacteria hybidizations were incubated at 45C. High G+C samples were incubated at 37C. After incubation slides were washed three times in 1XSET solution at 37C and examined using oil immersion epiflluorescent microscopy.

Results

White, opaque colonies appeared in 24 hours on all plates. Plate colonies matured in approximately 48 hours, becoming more tacky with time. Young and mature colonies all had a distinctiive smell of trimethylamine. The initial roll tube enrichments took about 3 days to grow and were much smaller, more wet colonies. However, all following reinoculates of tubes grew overnight.

Bacteria fell into the following morphological types: cocci (single cells, chains, clusters, diploids, tetrads), coccobacilli (single cells and diploids), and bacilli. Most cultures were dominated by cocci and coccobacilli (Table 1).

Cold enrichments were discontinued after the third streaking because the colonies were all identical to each other and not significantly different from the fresh samples. Similarly, the anaerobic plates were discarded because the growth on them was the same as that on the aerobic plates. As stated earlier, two women provided sample specimens, but after several streaking attempts the culture populations showed no difference among G+, M, and G plates. Subsequently, only DLH cultures were continued, the only exception being the blood agar plates.

Table 1. Cell Morphologies

Media	Description
G+ plate	short bacilli
M plate	small cocci
G plate	small cocci
Blood plates	small diplococci, short coccobacilli, elongate bacilli, short diplococcobacilli
G+ roll tube	short coccobacilli
M roll tube	short coccobacilli

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G roll tube cocci

*G+, 1% glycogen enriched w/hemin; M, .5% mucin enriched w/hemin; G, 1% glycogen; B, sheep's blood brain heart infusion

Only approximately 30% of the samples were catalase negative (Table 2). The oxidase test revealed almost an even division of oxidase negative and positive cells. The gram stain was positive for all samples tested.

Table 2.Catalase, Oxidase, and Hemolytic Test Results

Sample	Oxidase	Catalase	Hemolysis
1	+	+	B
2	-	+	B
3	-	+	B
4*	-	+	none
5*	+	+	B
6*	+	+	B
7	+	+	A
8*	+	+	none
9*	+	+	none
10*	+	+	none
11	-	+	none
12	-	-	none
13	-	-	none
14	-	-	none
15	-	-	none
16	-	-	none

B- beta hemolytic, A- alpha hemolytic
*AS blood plate sample, all others are DLH

In situ hybridization conducted on all cultures used high G+C, low G+C, and flavobacteria specific probes. Of the 8 samples tested for each probe, 2 of the low G+C showed positive (Table 3).

Table 3 Results of In Situ Hybridization

Sample	Fluorescence	Morphology
A	-	large & small cocci
B	-	diplococci
C	+	chained & single diplococci
D	-	short & long bacilli
E	+	diplococcobacilli
F	-	bacilli, cocci
G	-	diplo & chained cocci, bacilli
H	-	short bacilli

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Discussion

It was expected that most cells would be gram positive rods or cocci and that trimethylamine would be produced. Although the gram staining kit used (Difco) was not very accurate, it was largely indicative that the cells, mixed cultures and all, were gram positive. Catalase and Oxidase results were expected to be variable and were. These tests, along with the morphotyping, were done primarily to narrow down what the cells may be in preparation for the in situ hybridization. It is imprecise to comment on the production of trimethylamine because the HPLC was not accessible. Equipment modifications were needed that did not fit into the time period allotted for the experiment. However, it will suffice to say a distinct, pungent odor suggestive of trimethylamine was produced by all cultures. General comparisons between samples and pure solutions of cadaverine and putrescine support this assumption.

The cold enrichment technique was ineffective since it yielded no rod bacteria, although it has been recommended for cultivating Mobiluncus species. The possible explanations for this are that

1) the sample provider(s) were not infected
2) the bacilli were killed during the procedure
3) other opportunistic microbes overcrowded the target organisms

Another interesting phenomenon was the similarity between the aerobic and anaerobic cultures. This is most likely explained by the inability of the anaerobic jars to remain anoxic. At best the atmosphere was microaerophilic and therefore was unsuitable for obligate anaerobes as Mobiluncus and other cells.

In situ hybridization and lab analyses reveal that the positive samples probably contain Staphylococcus, Streptococcus, and Lactobacillus. Gram stain, catalase, oxidase, and probe data elimnated the possibility of there being Bacteroides, Gardnerella, Trichomonads, Neisseria, or Chlamydia.

Because of the similarity of morphotypes and lack of extraction methods, its difficult to positively identify species. Future efforts should focus on GC and HPLC methods for quantifying amines to better discern between the existing anaerobic biota.

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References

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