Survival of Gram positive anaerobic cocci on swabs and their isolation from the mouth and vagina

GILLIAN LF SMITH,*  CG CUMMING,†  PW ROSS*

From the *Department of Bacteriology, University Medical School, Edinburgh, and the †Department of Oral Medicine and Oral Pathology, Old Surgeons Hall, Edinburgh

SUMMARY The survival of Gram positive anaerobic cocci on plain cotton wool and albumin coated swabs held in various transport media was investigated. Results suggested that in most cases Amies’, Stuart’s and VMGII media do not offer any more protection to the bacteria than storing swabs dry in their containers. A technique was developed for the isolation and identification of Gram positive anaerobic cocci from the mouth and vagina, incorporating bicozamycin in the medium as a selective agent. Few strains were recovered from the oral cavity, but larger numbers were isolated from the vagina. Using a minimum number of antibiotic sensitivity and biochemical tests, including analysis of end products by gas-liquid chromatography, most isolates were identified to species level.

Recent improvements in laboratory techniques for the isolation and culture of anaerobic bacteria have greatly increased the recovery of these organisms from clinical samples, although service laboratories are still often presented with specimens that are inadequately taken or incorrectly transported to the laboratory. In such circumstances it may be impossible to isolate potentially pathogenic obligate anaerobes. In addition, the classification of many anaerobes is difficult, requiring numerous biochemical tests, the results of which may be equivocal. Gram positive anaerobic cocci are regarded as an important component of the commensal flora and are repeatedly isolated from clinical samples. Uncertainties about their classification induce most service laboratories to report only Gram positive anaerobic cocci. The purpose of this study was to evaluate the efficiency of various types of swab and transport media for the recovery of Gram positive anaerobic cocci and to develop a means of identifying to species level Gram positive anaerobic cocci isolated from oral and vaginal samples that would require a minimum number of tests.

Material and methods

SWAB TRIAL

Swabs and transport media Two types of swab were selected for this study: plain buffered absorbent cotton wool and albumin coated non-absorbent cotton wool (Exogen Ltd, Clydebank). Amies’, Stuart’s, and VMGII transport media were used.

Bacteria The strains of Gram positive anaerobic cocci used included six clinical isolates from the Royal Infirmary and Western General Hospital, Edinburgh, and four National Collection of Type Cultures strains previously identified to species level in the department of bacteriology, University of Edinburgh. They comprised three strains each of Peptostreptococcus anaerobius and Peptococcus magnus, two strains of Pc prevotii, and one strain each of Pc saccharolyticus and Pc asaccharolyticus. Modified Robertson’s cooked meat medium and 5% human blood agar plates were used for culture. All incubations were performed at 37°C for 48 hours (unless otherwise stated) using anaerobic jars (Baird and Tatlock Ltd; Don Whitley Scientific) according to the method of Collee et al.

Preparation of swabs Strains were incubated for 24 hours in 10 ml of prereduced cooked meat medium, and 0.02 ml was mixed in air with 0.08 ml sterile saline (0.85% w/v sodium chloride). This 0.1 ml volume was loaded on to each swab. Half blood agar plates were streaked with swabs either immediately before or after being held in their containers or in transport medium of up to six hours at both room temperature and at 4°C. At intervals of one, two, and six hours a set of swabs held under the various conditions was plated out, and plates were incubated anaerobically for 48 hours at 37°C. Colony forming units were counted and recovery rates calculated on the basis of the number of organisms originally
loaded on to the swabs. This was determined from dilutions of the parent culture by a spread plate viable count procedure.

Statistical methods comprised analysis of variance and t tests.

**Clinical Studies**

Fifteen dental students were randomly selected as sources of oral Gram positive anaerobic cocci; none was undergoing any form of periodontal treatment or had signs of upper respiratory tract infection. Vaginal strains were obtained from 20 asymptomatic women attending an antenatal clinic in the first trimester of pregnancy and 20 symptomatic women attending a sexually transmitted disease clinic. Patients who had been prescribed antibiotics within one month of the start of the study were excluded.

**Oral Cavity** Sterile dental floss in 3 cm lengths was passed through three interproximal sites, between the upper left canine and first premolar, the lower central incisors, and the lower right first molar and second premolar teeth. Each sample from the first five students was transferred into selective media consisting of 10 ml prerduced cooked meat medium containing 20 μg/ml neomycin, 16 μg/ml nalidixic acid, and 500 μg/ml bicozymycin. Floss from the next five students was transferred into 7 ml volumes of phosphate buffered saline, pH 7.4, containing 4 mg/ml L-cysteine hydrochloride and 3 mg/ml dithiothreitol. Samples on dental floss from the final five students were transferred into 7 ml proteose peptone yeast medium. Gingival crevices at the sample sites were probed to measure depth and assess gingival health; subsequent bleeding indicates local inflammation associated with gingivitis or periodontitis. An albumin coated swab was used to sample each student's throat and then replaced in its container; two saliva samples were obtained using Pasteur pipettes and transferred to prerduced phosphate buffered saline. All samples were transported to the laboratory within one hour. In the laboratory selective cooked meat broths containing samples were transferred to an anaerobic cabinet (Forma Scientific System Model 1024, Marietta, Ohio) for 48 hours of incubation at 37°C. Swabs were placed in the cabinet, transferred to selective cooked meat broths, and also incubated for 48 hours at 37°C. Samples in phosphate buffered saline and peptone yeast medium were sonicated for 30 seconds in an ultrasonic bath before transfer to the cabinet; 0.5 ml of each was inoculated with separate selective cooked meat broths and incubated for 48 hours at 37°C in the cabinet. After incubation the selective cooked meat broths were serially diluted in nutrient broth and 0.2 ml of 10^-4 and 10^-5 dilutions spread over the surface of 5% human blood agar plates with sterile glass spreaders. These plates were removed from the cabinet after 72 hours of incubation, giving a total of five days without exposure of organisms to atmospheric oxygen. Non-haemolytic grey, white or translucent colonies, ranging in size from a pinpoint to 2 mm in diameter were subcultured on to blood agar plates for 48 hours of parallel anaerobic and aerobic incubation at 37°C. Thirty such colonies could be placed on a single plate marked out in a grid pattern. Gram films were made of any colonies present on the anaerobic plates which had failed to grow on the aerobic plates. Organisms thought to be Gram positive anaerobic cocci were subcultured on to half blood agar plates with 5 μg vancomycin and 5 μg metronidazole discs. After 48 hours of incubation isolates sensitive to metronidazole and vancomycin were checked for purity, and 10 colonies were selected to inoculate 10 ml of cooked meat broth, which was incubated for 48 hours; this served as a stock culture for identification tests.

**Vagina** Samples were taken from the posterior fornix of 40 female subjects using plain cotton wool swabs. These swabs were left in their container and transferred to the anaerobic cabinet within two hours. They were placed in selective cooked meat broths and processed in an identical manner to the oral samples.

Throughout the study all media were reduced before inoculation either by storage under anaerobic conditions for 24-48 hours or, in the case of liquids, by holding at 100°C for 10-15 minutes.

**Identification of Isolates** Procedures for the identification of Gram positive anaerobic cocci were based on criteria outlined by Holdeman et al in the VPI laboratory manual. Tests comprised determination of acid production after 48 hours of anaerobic incubation in peptone yeast medium containing 1% (final concentration) glucose, indole, and coagulase production; formation of pigmented colonies on blood agar; and growth in the presence of 0.05% bile (only _Pc prevotii_ and _Pc indolicus_ grow in the presence of bile; Dr Watt, personal communication). Volatile and non-volatile short chain fatty acid end products of metabolism were analysed by gas-liquid chromatography on a column of 15% SP1220 on Chromosorb WAW at 147°C (Pye Unica 104 Chromatograph). In addition, isolates were subcultured on to blood agar plates to which 100 μg liquid (sodium polyanethol sulphonate) discs were added. Novobiocin discs (5 μg) were used to differentiate provisionally between peptostreptococci (sensitive) and peptococci (resistant).

**Results**

**Swab Trial** The mean recovery of 10 strains of Gram positive
anaerobic cocci from swabs inoculated on to blood agar immediately after loading was 9.5% of the initial inoculum from the cotton wool and 13.2% from the albumin coated swabs. Mean immediate recoveries calculated from individual species were 8.7% for cotton wool and 12.2% for albumin swabs (Table 1).

Tables 1 and 2 summarise the recovery from swabs stored in and without transport media for periods of up to six hours at either room temperature or 4°C. There was no important difference in the recovery of organisms from either type of swab held without transport medium at room temperature for two hours, but the highest mean recovery at six hours was obtained from albumin coated swabs stored at 4°C. Of the three transport media tested, only Amies' compared favourably with "dry" swabs. In general, if Gram positive anaerobic cocci were held on swabs for longer than two hours a considerably decreased recovery rate resulted compared with immediate, one hour, and two hour recoveries.

**RECOVERY FROM GRAM POSITIVE ANAEROBIC COCCI FROM CLINICAL MATERIAL**

Tables 3, 4, and 5 show the numbers and species of Gram positive anaerobic cocci from the oral cavity and vagina. Samples obtained using dental floss yielded 15 isolates from 24% of sites. All but one of these isolates were identified as *Pc magnus*. Five of the 15 throat swabs yielded growth of Gram positive anaerobic cocci, and all were identified as *Pc magnus*. Gram positive anaerobic cocci were not isolated from saliva. Other organisms isolated from the mouth included facultative and aerobic Gram positive cocci and pleomorphic rods, as well as Gram negative anaerobic cocci. The dental students examined had a high standard of oral hygiene; all gingival crevices were <3-5mm in depth, and none of these sites bled after probing.

Compared with the oral cavity, samples from the vagina contained higher proportions of Gram positive anaerobic cocci, which were isolated from 55% of antenatal and 80% of sexually transmitted disease samples, respectively. The Gram positive anaerobic cocci most often encountered were *Ps anaerobius* (32% of sexually transmitted disease isolates, 21% of antenatal isolates), *Pc prevotii* (28% and 29%, respectively), and *Pc magnus* (15% and 27%, respectively). Laboratory tests failed to speculate nine isolates of Gram positive anaerobic cocci, two of which could not be assigned to genus level, but sensitivity of metronidazole and vancomycin indicated that they were Gram positive anaerobic cocci. Procedures for isolation and identification required a minimum of 12 days following receipt of samples.

**Discussion**

The critical factors entailed in the survival of clinically important bacteria on swabs and their subsequent recovery have been well documented. Collee et al described mean recoveries of 3–5% of anaerobic bacteria from swabs inoculated on to blood agar plates immediately after loading. They concluded that the low recovery rates were due primarily to retention of bacteria on swabs rather than to the toxic effects of swab components or exposure to atmospheric oxygen. In their study it was emphasised that such test conditions did not adequately reproduce the in vivo conditions that inevitably affect the isolation of bacteria from infected sites. In this study mean recovery rates from both cotton wool and albumin coated swabs were higher than those recorded by Collee et al.

Several transport media suitable for anaerobic bacteria have been described. Holbrook et al, however, found VMGII suitable for the storage of gingival crevice samples containing *Bacteroides melaninogenicus*. On the basis of the present results VMGII transport medium cannot be recommended for the storage of Gram positive anaerobic cocci. Similarly, the use of Stuart's medium cannot be advocated, although it has been used previously with some success in the recovery of anaeobes from the female genital tract. We concluded that plain cotton wool or albumin coated swabs were most efficient in the recovery of Gram positive anaerobic cocci held in their containers for up to two hours at room temperature. None of the transport media tested improved the recovery rate.

Anaerobic organisms are important components of the normal flora of both the mouth and the vagina, but, occasionally, these bacteria are associated with both local and disseminated infections. In the present study the low numbers of Gram positive anaerobic cocci isolated from oral samples may be a reflection of the excellent oral health of the subjects chosen. A comparative study of samples from patients man-
after one, two, and six hours.

Table 2  Mean percentage recovery of 10 strains of Gram positive anaerobic cocci at room temperature (RT) and 4°C after one, two, and six hours

<table>
<thead>
<tr>
<th>Swab</th>
<th>Storage of swabs</th>
<th>Time (hours)</th>
<th>Dry RT</th>
<th>Dry 4°C</th>
<th>Amies' RT</th>
<th>Amies' 4°C</th>
<th>Stuart's RT</th>
<th>Stuart's 4°C</th>
<th>VMGII RT</th>
<th>VMGII 4°C</th>
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<tr>
<td>Cotton wool:</td>
<td></td>
<td>1</td>
<td>8.7</td>
<td>6.7</td>
<td>3.4</td>
<td>6.6</td>
<td>2.9</td>
<td>1.4</td>
<td>3.3</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>6.3</td>
<td>3.7</td>
<td>6.0</td>
<td>6.8</td>
<td>1.3</td>
<td>2.4</td>
<td>3.3</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>1.2</td>
<td>3.8</td>
<td>6.0</td>
<td>4.1</td>
<td>0.6</td>
<td>1.5</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>5.4</td>
<td>3.7</td>
<td>6.0</td>
<td>5.8</td>
<td>1.6</td>
<td>1.8</td>
<td>2.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Albumin:</td>
<td></td>
<td>1</td>
<td>9.3</td>
<td>4.2</td>
<td>8.8</td>
<td>4.7</td>
<td>3.1</td>
<td>4.4</td>
<td>4.5</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>5.5</td>
<td>3.9</td>
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<td>0.5</td>
<td>3.2</td>
<td>6.6</td>
<td>5.9</td>
<td>0.5</td>
<td>2.9</td>
<td>2.8</td>
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<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>5.1</td>
<td>3.4</td>
<td>7.9</td>
<td>8.1</td>
<td>2.1</td>
<td>3.5</td>
<td>4.0</td>
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RT = room temperature

Table 3  Isolation of Gram positive anaerobic cocci from oral cavity

<table>
<thead>
<tr>
<th>Sample</th>
<th>No of samples</th>
<th>No positive for Gram positive anaerobic cocci (%)</th>
<th>No of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interproximal</td>
<td>45</td>
<td>11 (24)</td>
<td>15</td>
</tr>
<tr>
<td>Saliva</td>
<td>30</td>
<td>0 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Throat swabs</td>
<td>15</td>
<td>5 (33)</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>16 (18)</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 4  Isolation of Gram positive anaerobic cocci from vagina

<table>
<thead>
<tr>
<th>Sample</th>
<th>No of samples</th>
<th>No positive for Gram positive anaerobic cocci (%)</th>
<th>No of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sexually transmitted disease clinic</td>
<td>20</td>
<td>16 (80)</td>
<td>89</td>
</tr>
<tr>
<td>Antenatal clinic</td>
<td>20</td>
<td>11 (55)</td>
<td>56</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>27 (68)</td>
<td>145</td>
</tr>
</tbody>
</table>

Table 5  Species of Gram positive anaerobic cocci from oral cavity and vagina

<table>
<thead>
<tr>
<th></th>
<th>Interproximal</th>
<th>Throat</th>
<th>Antenatal (%)</th>
<th>Sexually transmitted disease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptostreptococcus anaerobius</td>
<td>14</td>
<td>7</td>
<td>12 (21)</td>
<td>28 (32)</td>
</tr>
<tr>
<td>Peptococcus magnus</td>
<td></td>
<td></td>
<td>15 (27)</td>
<td>13 (15)</td>
</tr>
<tr>
<td>Peptococcus asaccharolyticus</td>
<td></td>
<td></td>
<td>2 (4)</td>
<td>15 (17)</td>
</tr>
<tr>
<td>Peptococcus prevotii</td>
<td>16</td>
<td>29</td>
<td>16 (29)</td>
<td>25 (28)</td>
</tr>
<tr>
<td>Peptococcus saccharolyticus</td>
<td></td>
<td></td>
<td>1 (1)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Peptostreptococcus micros</td>
<td>1</td>
<td></td>
<td>1 (1)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Peptococcus spp</td>
<td>7</td>
<td>13</td>
<td>7 (13)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Unidentified</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ifesting periodontal pathology would be valuable. Few Gram positive anaerobic cocci were recovered, so it is difficult to be dogmatic about which of the three transport media was superior. It was easier to place the floss or swabs directly into selective media.

Although peptone yeast medium is a rich source of nutrients for many bacteria, samples were held in it for less than one hour, and it may be that this protein containing medium affords some protection for delicate organisms. Failure to isolate Gram positive anaerobic cocci from saliva was initially surprising after the findings of Socransky and Manganiello,21 but we conclude that our results indicate a low incidence of Gram positive anaerobic cocci in the healthy oral cavity.

It is impossible to say whether or not several isolates of the same species recovered from a single specimen represent the same or different isolates. This is a reflection of the failure of Gram positive anaerobic cocci to display distinctive colonial morphologies; several very similar colonies picked from primary culture plates often proved to be different species of Gram positive anaerobic cocci.

The combination of selective agents had previously been tested with stock strains of Gram positive anaerobic cocci held in our laboratory and did not interfere
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with their growth. Neomycin is normally used at a concentration of 70–75 μg/ml in selective media, but there is some evidence of synergy by bicozamycin, which might prove inhibitory to some Gram positive anaerobic cocci. Neomycin was used, therefore, at a concentration of 20 μg/ml.

A greater number and wider variety of isolates were found in vaginal samples in comparison to oral samples. Our findings are in broad agreement with those of Bartlett and Polk. A much higher proportion of symptomatic women attending a sexually transmitted disease clinic carried Gram positive anaerobic cocci in the vagina compared with asymptomatic pregnant women. Half of the symptomatic group were culture negative for "classical" sexually transmitted pathogens. These findings bear comparison with the evidence produced by Spiegel et al and Taylor et al, which strongly suggests a pathogenic role for anaerobes in non-specific vaginitis. In this study failure to speciate an isolate was attributable to poor growth, either on subculture with novobiocin and liquid discs or in fermentation tests; repeated cultures failed to improve growth. All the Gram positive anaerobic cocci isolated conformed to the definition of Watt and Jack in that they would not grow in air, nor in air plus 10% carbon dioxide after seven days of incubation at 37°C.

The taxonomy of Gram positive anaerobic cocci is far from satisfactory and is constantly under review. Kilpper-Balz and Schleifer proposed the transfer of *Pc saccharolyticus* to the genus *Staphylococcus*; Cato suggested that *Ps parvulus* be placed in the genus *Streptococcus*; Ezaki et al. proposed the transfer of *Pc indolicus*, *Pc magnus*, *Pc saccharolyticus*, and *Pc prevotii* to the genus *Peptostreptococcus* on the basis of DNA–DNA homologies and cellular fatty acid composition. Thus service laboratories are reluctant to undertake time consuming tests requiring specialised equipment for GLC to identify Gram positive anaerobic cocci according to schemes that may be taxonomically invalid. The use of novobiocin discs to distinguish between *Peptococcus* and *Peptostreptococcus* is not infallible. In such a case the only means of distinguishing *Pc magnus* from *Ps micros* is by this organism's small cell size in Gram films, but Hare observed that even this is not consistent. Watt et al. recently proposed a simplified scheme ideal for service laboratories based on glucose fermentation and liquid susceptibility, although the authors recognise its "doubtful taxonomic validity".

The present scheme succeeded in identifying most isolates using a greatly reduced number of tests, but GLC analysis of acid end products was required to place Gram positive anaerobic cocci in currently accepted species. The development of a universally accepted classification scheme for Gram positive anaerobic cocci with a sound taxonomic foundation is of the highest priority, so that the pathological and epidemiological importance of these organisms can be understood.

We thank Dr B Watt for supplies of bicozamycin, Dr A McMillan of the department of genitourinary medicine, and the staff of the Simpson antenatal clinic, for supplying vaginal specimens. We also thank Mr R Brown and Mrs Hannah Lough for expert technical help and Mr B Adams for statistical help.

GLF Smith acknowledges support of a Faculty of Medicine fellowship.

References


Requests for reprints to: Dr PW Ross, Department of Bacteriology, University Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland.
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G L Smith, C G Cumming and P W Ross

doi: 10.1136/jcp.39.1.93

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