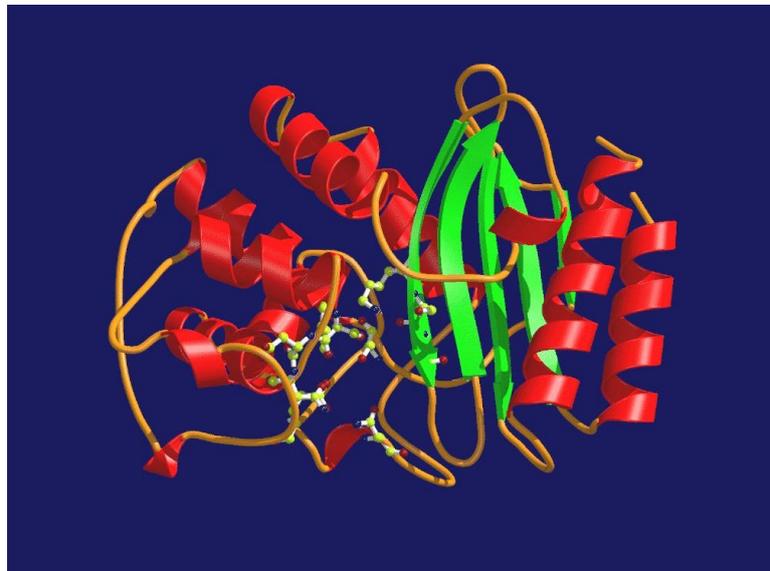


# $\beta$ -lactamase-producing bacteria in periodontal health and disease

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<http://www.antibioresistance.be/betalactamases.html>

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## **Abstract**

To determine the extent of  $\beta$ -lactamase-producing bacteria in the gingival sulcus, subgingival biofilm samples of 20 healthy individuals aged between 28 and 64 years were investigated.  $\beta$ -Lactamase activity was assessed by the chromogenic nitrocefin method and  $\beta$ -Lactamase-positive isolates were characterized using commercial diagnostic kits and partial sequencing of the 16S rRNA gene. Enzyme activity was detected in 35 % of the individuals. The most prominent  $\beta$ -lactamase-producing organisms belonged to the anaerobic genus *Prevotella*. Other bacteria evident in our samples were *Fusobacterium spp.*, *Capnocytophaga sp.*, *Streptococcus mutans*, and *Campylobacter rectus*. In comparison to the prevalence of  $\beta$ -lactamase-producing subgingival bacteria in patients with refractory periodontitis, the extent of enzyme activity in the healthy gingival sulcus was reduced by ~50 %.

## Introduction

Excessive use of antibiotics over the last 50 years has led to many important pathogenic bacteria developing resistance towards antibiotics. The normal bacterial flora in the mouth, the so called “nice” bacteria, may develop antibiotic resistance, especially after multiple previous antibiotic treatments (Marsh & Martin 4<sup>th</sup> ed. 1999). This is not necessarily significant, but problems may arise when bacteria from the normal flora transmit their resistance genes to pathogenic bacteria they get in contact with.

Evidence exists that antibiotic resistance has increased in the oral flora over the last 10 to 15 years (Walker CB. 1987 and 1996). The most commonly prescribed antimicrobials worldwide are the  $\beta$ -lactam antibiotics, especially penicillin (Danziger LH., Pendland SL. 1995). One important mechanism of bacterial resistance towards penicillin is the production of  $\beta$ -lactamase which is an enzyme capable of hydrolyzing the penicillin and destroying its antimicrobial properties (Frère JM. 1995). The emergence of  $\beta$ -lactamase-producing bacteria may protect non-producing bacteria against the  $\beta$ -lactam antibiotics. This may lead to therapeutic failure.

Antimicrobial resistance determinants may be exchanged between bacteria sharing common ecological niches. An established oral biofilm structure provides an optimal environment for the exchange of genetic material between cells. Several genetic elements are involved in the assembly and spread of antimicrobial resistant determinants. The development of antimicrobial resistance has led to the discovery of many natural mobile elements, including conjugative transposons and conjugative plasmids. Conjugative transposons are chromosomally located non-replicative genetic elements that encode functions necessary for their own excision and intercellular transfer. Conjugative plasmids are extrachromosomal replicative DNA forms that can encode a large variety of important genes, including resistance genes. Conjugative plasmids can transfer into and replicate within a variety of different species. Having entered into a new genus on broad host range plasmids, resistance determinants can readily transfer onto more frequently transferable plasmids to increase their movement through the new genus. Plasmids may also integrate into the chromosome of the recipient strains, potentially increasing the stability of the genetic information they carry.

$\beta$ -lactamases have been reported as an increasingly important problem in the microflora associated with oral infection, particularly periodontitis, in many countries. Several investigators have reported  $\beta$ -lactamase

production in periodontal isolates of *Prevotella* spp. (Bernal LA et al. 1998, Dubreuil L et al. 2003, Fosse T et al. 1999, Handal T et al. 2003, Herrera D et al. 2000, Asikainen S et al. 1999, Valle G et al. 1998, van Winkelhoff AJ et al. 1997). Resistance has also been described in periodontal pathogens such as *Fusobacterium* spp., *Capnocytophaga* spp. and in oral superinfecting *Enterobacteriaceae* and *Staphylococci* (Foweraker JE et al. 1990, Goldstein EJC et al. 1995, Kinder SA et al. 1986, Nyfors S et al. 1999 and 2003, Roscoe DL et al. 1992). Linkage of several classes of antibiotic resistance genes on conjugative elements and gene transfer between different species have been demonstrated in oral *Prevotella* (Guiney DG et al. 1990, Walker CB et al. 1997). Antimicrobial treatment of dental infections may promote the emergence of bacterial resistance, both in the diseased sites and in the normal oral flora. The oral microflora may therefore act as a gene pool for antibiotic resistance, which may be acquired from and passed onto transient colonizers of the site.

Currently, little is known about the presence and variety of  $\beta$ -lactamase-producing bacteria in the normal oral flora associated with periodontal health. In the present study, we hypothesized that the prevalence of  $\beta$ -lactamase-producing bacteria in healthy individuals would be less than that in patients with periodontal disease. The purpose of this study was to assess the prevalence and variety of  $\beta$ -lactamase-producing bacteria in the gingival sulcus of healthy individuals in Norway. A second objective was to compare the results to two similar studies describing the  $\beta$ -lactamase-producing microflora in periodontal disease in Norway and the USA (Handal et al. 2003 and 2004).

## **Material and methods**

### ***Description of the study material***

We investigated volunteers with a very good standard of oral hygiene without any sign of periodontal disease. Twenty healthy women and men aged between 28 and 64 years were included in the study. None of the individuals had taken any antibiotics in the last three months prior to sampling. Biofilm samples were collected from four teeth (typically 14, 23, 34 and 43) by means of two sterile paper points per tooth. The subgingival sites had probing depths  $\leq 2$ mm, and there were no bleeding on probing. The paper points were introduced into the gingival sulcus for 15 sec, after that, placed immediately in prereduced anaerobically sterilized Dental Transport Medium (Anaerobic system, Morgan Hill, Calif.). This transport system is adequate for the transport and recovery of fastidious oral anaerobic bacteria, including *Tannerella forsythia* and *Porphyromas gingivalis*.

### ***Sample processing and culture***

In the laboratory, the sealed tubes with the microbiological samples were agitated for 10 sec on a whirly mixer (Labinco, Breda, the Netherlands). Three serial 10-fold dilutions of the transport fluid medium were made in one-quarter strength prereduced anaerobically sterilized (PRAS) Ringer's solution supplemented with 0.05% l-cysteine free base (Sigma, St. Louis, MO). A VPI Anaerobic Culture System (Bellco, Vineland, NJ) was used to flush the tubes continuously with an anaerobic gas mixture (90% N<sub>2</sub>, 5% H<sub>2</sub>, 5% CO<sub>2</sub>) during seeding. Each dilution, while kept in the Anaerobic Culture System, was pipetted out in volumes of 0.1 ml onto nonselective trypticase soy agar plates supplemented with 5% defibrinated human blood, hemin (5 mg/ml) and menadione (0.05 mg/ml). For isolation of  $\beta$ -lactamase-producing organisms relatively resistant to amoxicillin, blood agar plates were supplemented with 3  $\mu$ g/ml amoxicillin.

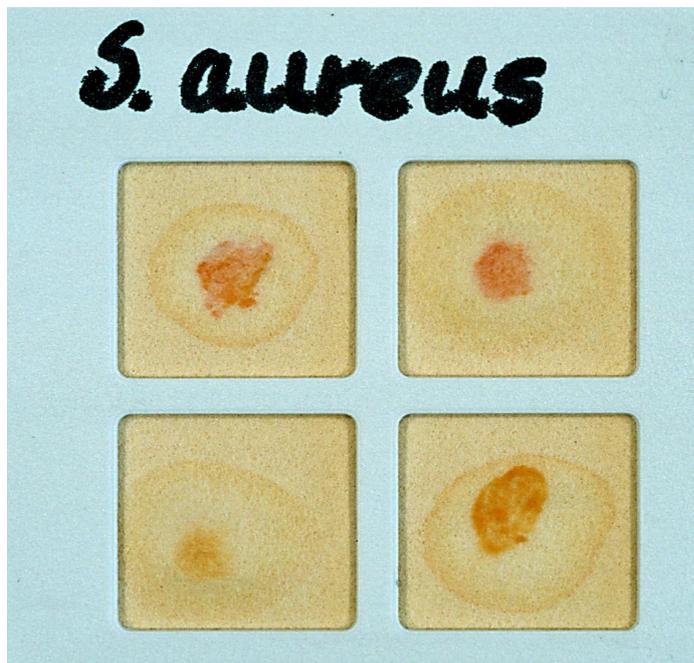
**All morphotypes of bacteria on plates were subcultured and then tested for  $\beta$ -lactamase activity.**



**Biofilm sample cultured on blood agar.**

### ***Testing of $\beta$ -lactamase activity***

$\beta$ -lactamase production was assessed using chromogenic nitrocefin-impregnated disks (BBL™ DrySlide™ Nitrocefin, Becton Dickinson, Sparks, MA) (30).  $\beta$ -lactamase-positive and -negative strains of *Staphylococcus aureus*, provided by the Microbiology Laboratory at the National Hospital, Oslo, were included as controls.  $\beta$ -lactamase-positive bacteria were preserved in 1.5 ml Todd-Hewitt solution at -20°C and further characterized.



**Dryslide Nitrocefin Test; Positive: pink; Negative: yellow**

## ***Identification of $\beta$ -lactamase-producing isolates***

Preliminary identification of pure cultures was based on aerotolerance, colony and cell morphology, colony pigmentation and gram-staining of cells. Enzymatic/biochemical profiling relied on commercial diagnostic kits designed for identification of a number of different microorganisms (API, bioMerieux, Marcy-l'Etoile, France). The preparation and incubation of the kits were carried out according to the manufacturer's recommendations. Reading of the kits occurred automatically in an ATB reader (API, bioMerieux). The results of the reactions, transferred into a numerical code, were treated in a database system for identification (API Plus, bio-Merieux).

To confirm species identification, partial sequencing of the 16S rRNA gene was performed. One loop of bacterial culture was suspended in 450  $\mu$ l of distilled sterile water, and DNA was extracted using Magnatrix 1200 (Magnetic Biosolutions, Stockholm, Sweden) utilizing the MagAttract DNA Mini M48Kit (Qiagen, CA). The obtained solution of DNA could be used without dilution. DNA (1  $\mu$ l of supernatant) was amplified in a reaction mixture consisting of 5  $\mu$ l of 10 x polymerase chain reaction (PCR) buffer, 4  $\mu$ l of 10  $\mu$ M total deoxynucleotidetriphosphates, 1  $\mu$ l of 10  $\mu$ M primer PA and PD (MWG-Biotech, GmbH, Ebersberg, Germany) (28), 37.75  $\mu$ l of distilled H<sub>2</sub>O, and 0.25  $\mu$ l of Amplitaq DNA polymerase. PCR conditions included 30 cycles of denaturation at 95°C for 40 s, annealing at 58°C for 1 min, and extension at 72°C for 40 s. DNA amplicons (7  $\mu$ l) were purified in a mixture containing 1  $\mu$ l exonuclease and 1  $\mu$ l shrimp alkaline phosphatase, and incubated for 15 min at 37°C and 15 min at 80°C, as recommended by the manufacturer (Amersham Biosciences, Cleveland, OH). The purified product was sequenced on both strands using the primers PB and PC (MWG-Biotech, GmbH) (28) and the Big-Dye Terminator mix (Applied Biosystems, Foster City, USA), according to the manufacturer's instruction. The elongation products were then applied into Long-Ranger gel 5.0% (Cambrex, Rockland, ME), and the DNA sequences were read automatically with an ABI Prism 377 DNA sequencer and entered into the AutoAssembler™ software vs.

2.1program(Applied Biosystems). The sequences were analyzed with the Blast 2.1 program from the GenBank Online Service.

## **Results**

At least one isolate with  $\beta$ -lactamase activity was detected in 7 (35%) of the 20 individuals, and a total of 16  $\beta$ -lactamase-producing bacterial morphotypes were found in the sulcus samples from these individuals. The  $\beta$ -lactamase-positive isolates identified are listed in Table 1.

The most prominent  $\beta$ -lactamase-producing species were anaerobic gram-negative rods belonging to the genus *Prevotella*. These species were found in all seven individual samples testing positive for  $\beta$ -lactamase. More than one species of *Prevotella* was found in the sample from two individuals: no. 11, *Prevotella intermedia* and two other *Prevotella* spp. that could not be identified at the species level; no. 12, *P. bivia* and another *Prevotella* sp.

Other enzyme-producing strains were *Fusobacterium nucleatum* (two strains), *Capnocytophaga* sp. (one strain), *Campylobacter rectus* (one strain) and *Streptococcus mutans* (one strain).

When comparing the API system and partial sequencing of the 16S rRNA gene, there was no accordance at the species level for any of the individual strains. However, accordance at the genus level was observed for *Prevotella* spp. and *Streptococcus* sp.

**Table 1: Prevalence and identity of  $\beta$ -lactamase producing isolates:**

| Individual No. | $\beta$ -lactamase +/or - | Identity<br>16S rRNA<br>(%homology)                                  | Identity<br>API<br>(%reliability)  |
|----------------|---------------------------|--|--|
| 1              | -                         |  |  |
| 2              | -                         |  |  |
| 3              | +                         | <i>Prevotella dentalis</i> (100)<br><i>Campylobacter rectus</i> (99) | <i>Prevotella buccae</i> (*)<br>ND   |
| 4              | -                         |  |  |
| 5              | -                         |  |  |
| 6              | -                         |  |  |
| 7              | -                         |  |  |
| 8              | -                         |  |  |
| 9              | +                         | <i>Prevotella</i> sp. (95)   | <i>P. buccae</i> (*)   |
| 10             | -                         |  |  |
| 11             | +                         | ND<br><i>Prevotella</i> sp. (99)<br><i>Prevotella</i> sp. (99)<br>ND | <i>P. intermedia</i> (*)<br><i>P. buccae</i> (*)<br><i>Prevotella bivia</i> (43)<br><i>F. nucleatum</i> (63) |
| 12             | +                         | ND<br><i>Prevotella</i> sp. (97)<br>ND                               | <i>P. bivia</i> (*)<br><i>P. loescheii</i> (58)<br><i>Capnocytophaga</i> sp.(*)                              |
| 13             | -                         |  |  |

|           |          |                                   |   |
|-----------|----------|-----------------------------------|---|
| <b>14</b> | <b>+</b> | <i>S. mutans</i> (99)<br>ND<br>ND | <i>Streptococcus mitis</i> (77)<br><i>F. nucleatum</i> (97)<br><i>Prevotella loescheii</i> (85) |
| <b>15</b> | <b>-</b> |                                   |   |
| <b>16</b> | <b>+</b> | ND                                | ND  |
| <b>17</b> | <b>+</b> | ND*<br>ND*                        | ND*<br>ND*  |
| <b>18</b> | <b>-</b> |                                   |   |
| <b>19</b> | <b>-</b> |                                   |   |
| <b>20</b> | <b>-</b> |                                   |   |

\*: % reliability not given

ND: not determined

ND\*: not determined by the two identifying methods; anaerobic pigmented rods

## Discussion

The results of this study revealed that 35 % of a subpopulation of healthy individuals in Oslo, Norway, harboured  $\beta$ -lactamase-producing bacteria in the gingival sulcus. Previous studies involving  $\beta$ -lactamase-producing *Eschericia coli* and *Klebsiella pneumoniae* in asymptomatic healthy individuals resulted in a prevalence of 13 % (Kader AA et al. 2007). The authors concluded that that carriage of commensal  $\beta$ -lactamase-producers in preschool-age healthy children mostly reflected exposure to contamination in the family environment.

Bacteria belonging to the genus *Prevotella* were the most commonly detected in the present material, as it was found in five of the seven individuals testing positive for  $\beta$ -lactamase activity. *Prevotella* has previously been strongly associated with  $\beta$ -lactamase production (Bernal LA et al. 1998, Dubreuil L et al. 2003, Fosse T et al. 1999, Handal T et al. 2003, Herrera D et al. 2000, Asikainen S et al. 1999, Valle G et al. 1998, van Winkelhoff AJ et al. 1997).

Other bacterial findings included *Campylobacter rectus* which has been regarded as a putative periodontal pathogen. A few reports have, however, concluded that this bacterium also reside in healthy sites as a part of the normal oral flora (Dahlén G et al. 1992, Gmur R et al. 1994). Gingival sulcus bacteria may possibly function as principal reservoir(s), and if they forcibly lead to periodontal destruction, their resistance profiles may compromise antimicrobial treatment. Periodontal bacteria represent predominantly opportunistic microorganisms which may have a natural habitat in the gingival sulcus.

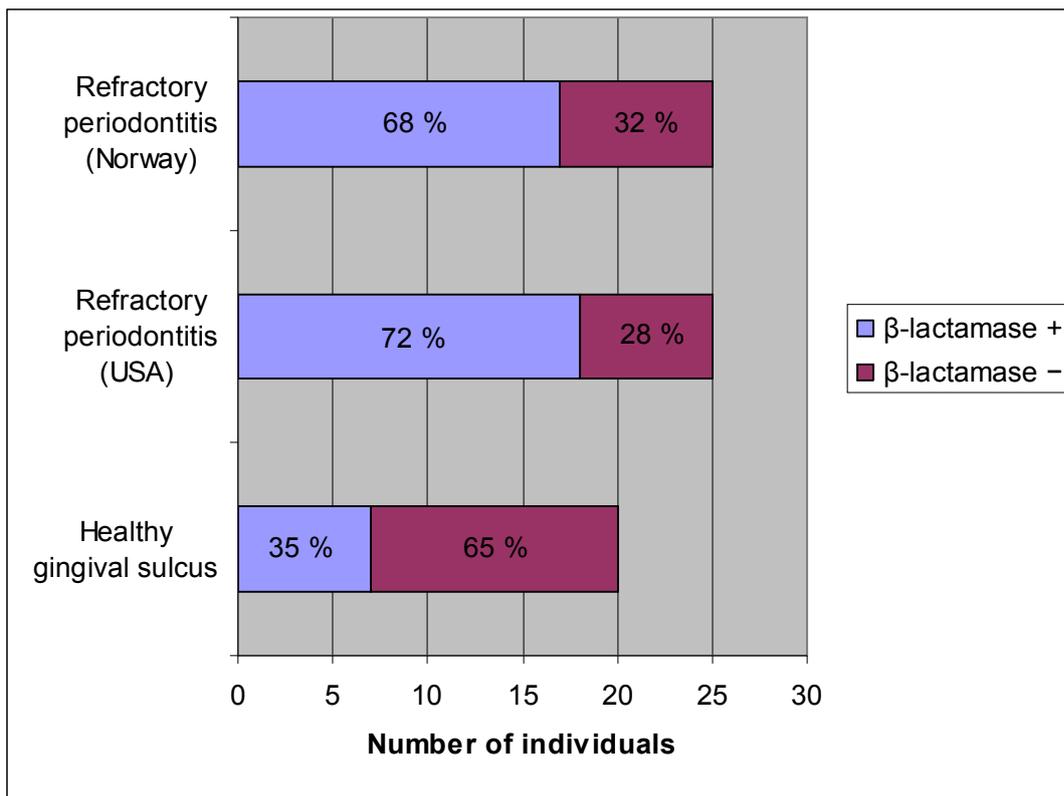
*F. nucleatum* was found in two individuals. Earlier findings link this bacterium to both periodontitis and the emergence of antibiotic resistance (Adriana et al. 2007). Nyfors S et al. (2003) reported that penicillin resistance due to  $\beta$ -lactamase production was surprisingly common among oral commensal bacteria in childhood. The prevalence of infants harbouring penicillin-resistant *F. nucleatum* due to  $\beta$ -lactamase production increased with age and usage of antimicrobial agents during the first year of life.

Another bacterium, found the present study was *S. mutans*. This bacterium has previously been found to be producing  $\beta$ -lactamase when taken from carious teeth (Fani MM et al. 2007).

A second objective in our study was to compare the results to two similar studies describing the  $\beta$ -lactamase-producing microflora in periodontal disease in Norway and the USA (Handal et al. 2003 and 2004) (Figure 1). The methods used in those studies and the present study

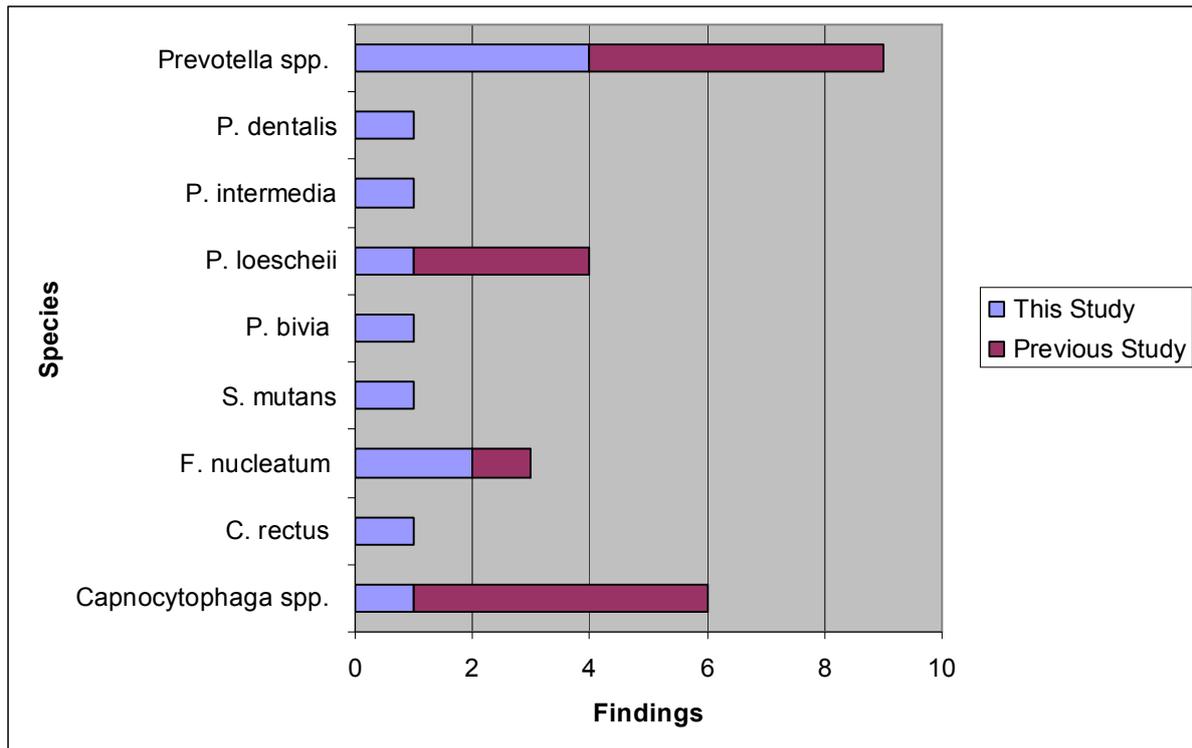
were similar. The authors reported prevalences of 68 % and 72 % of patients with refractory periodontitis in Norway and the USA, respectively. These findings are in accordance with our hypothesis, that the prevalence of  $\beta$ -lactamase-producing bacteria in healthy individuals would be less than in persons with periodontal disease. Previous studies in the USA reported prevalences of 76 % and 64 % in untreated periodontitis patients and maintenance patients, respectively (Legg JA et al. 1986, Walker CB et al. 1987). High prevalences of  $\beta$ -lactamase-producing bacteria have also been reported in patients suffering from chronic periodontitis (Herrera D et al. 2000, Legg JA et al. 1986) and rapidly progressive periodontitis (Fosse T et al. 1999).

**Figure 1: Comparison of the prevalence of  $\beta$ -lactamase positive strains between health and disease:**



A higher proportion of *Prevotella* was found in healthy compared to diseased sites (Fig. 2). Not surprisingly, no *Enterobacteria*, *Burkholderia* spp., *Ralstonia* spp. and *Bacillus* spp. were found in the healthy gingival sulcus compared to sites with refractory periodontitis.

Figure 2: Comparison of  $\beta$ -lactamase-producing bacteria in this study and a previous study:



In summary,  $\beta$ -lactamase production was detected in several different bacteria from the gingival sulcus of healthy individuals in Norway. The prevalence was reduced by approximately 50% when compared to sites exhibiting refractory periodontitis. Accordingly,  $\beta$ -lactamase-producing bacteria are more prominent in individuals with periodontal disease. However, healthy individuals harbour  $\beta$ -lactamase-producing bacteria in their normal oral microflora. This may be important if these individuals need antibiotic treatment in the future, either as treatment for periodontitis or other oral disease. The *Prevotella* group of bacteria seems to dominate both in health and disease, however, several other residents in the oral cavity exhibit resistance genes. This probably reflects previous antibiotic consumption or contamination in the family and milieu. This study may therefore be a further strengthening of the theory that the oral flora can act as a  $\beta$ -lactamase gene reservoir that can possibly spread to transient colonizers of the site (Handal et. al 2005).

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