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Original Research

Molecular Detection of the Predatory Bacterium *Bdellovibrio bacteriovorus* from Dental Biofilms

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Abstract

Introduction: The oral microbiome is a complex community whose composition results from multiple interactions among different microorganisms and with the host. Predatory prokaryotes are recognized as important balancing factors in different ecosystems. Among them *Bdellovibrio bacteriovorus* received special attention for its strong predatory behaviour against many human pathogens in the absence of any toxic or pathogenic effect, so that it was proposed as a live probiotic/antibiotic agent. This work aimed to evaluate if *B. bacteriovorus* is detected from samples of human oral and dental biofilm. **Materials and Methods:** Samples of oral and dental biofilm were obtained from 20 adults of both sexes and processed for extraction of metagenomic DNA, to be used as templates for *B. bacteriovorus*-specific PCR reactions. Specificity of amplification products was confirmed by sequencing. **Results:** All 20 dental biofilm samples and 12 of 20 (60%) oral biofilm samples were resulted PCR positive. The sequences of 17 of 32 PCR products (53.1%) showed 100% identity with the reference sequence; the sequences of 11 of 32 PCR products (34.4%) showed ≥99% identity, while the remaining 4 products (12.5%) showed identities ranging between <99% and ≥97%. **Conclusions:** This is the first survey specifically reporting the presence of *B. bacteriovorus* in the human oral cavity and suggests that bacterial predation is a relevant balancing factor for the oral microbiota. Demonstration that *B. bacteriovorus* is able to colonize the oral cavity gives strength to proposals of its use as a probiotic/antibiotic in the prevention/treatment of selected oral diseases.

Keywords: *Bdellovibrio bacteriovorus*, dental biofilm, oral biofilm, predatory bacteria

Introduction

The oral microbiome is an array of incredibly complex microbial communities colonizing the different niches of the oral cavity and is formed by various arrangements of about 200 predominant taxa and other 500 less represented taxa.[1-2]

Complex interactions among these microorganisms and between them and the host significantly impact oral health, the onset of different oral diseases,[2-6] and even systemic diseases.[7-9]

Many different factors allow or contrast the survival and growth of bacteria in natural ecosystems; these include host defences, naturally produced antibiotics, bacteriocins, natural antimicrobic peptides, and predation by bacteriophages and predatory prokaryotes.[10-12]

The role of predatory prokaryotes as balancing factors in different natural ecosystems and hosts has received lot of attention in recent years.[13,14]

Predatory bacteria are prokaryotes who live by selectively killing some species of cohabitant bacteria by means of a variety of killing strategies.[11,15] Available data suggest that these microorganisms are among the most important factors in bacterial selection and mortality in some ecosystems, so that predation has been proposed as an evolutionary driving force.[15]

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Among predatory bacteria, *Bdellovibrio bacteriovorus* has received special attention: it is a small and highly motile Gram-negative facultative anaerobic/aerobic bacterium that belongs to the group of Bdellovibrio-and-like organisms (BALO) of the class -Proteobacteria.[16] It is widely distributed in different environments and grows by selectively predating other Gram-negative bacteria,[17] although it does not disdain to attack and kill also some Gram positives.[18,19]

The life cycle of *B. bacteriovorus* alternates between two distinct forms: a free-living motile form searching for its prey and an intracellular predating form growing within the prey. [17]

The niche where *B. bacteriovorus* usually replicates is the periplasmic space of its host cell, which is finally eventually lysed to begin a new cycle of predation. [17]

It was recently shown that *B. bacteriovorus* is part of the human gut microbiota where it is directly involved in maintaining eubiosis.[20]

Among the bacterial preys that are actively killed by *B. bacteriovorus*, several important oral pathogens are included.[21-23]

Considered the efficient predatory activity on many relevant human pathogens and its lack of toxic or pathogenic effects on cultured cells and in animal models, *B. bacteriovorus* was proposed and positively tested as alternative/adjunctive anti-infective agent in the treatment of different human infections,[24-26] including periodontitis.[27]

The possibility to successfully use some predatory bacterial species, and *B. bacteriovorus* in particular, as a live probiotic/antibiotic agent in the treatment of selected oral infections (including periodontitis and periimplantitis) largely depends on the possibility that these bacterial species are adapted to colonize the human oral cavity.

This work was consequently aimed to evaluate if *B. bacteriovorus* is detected from samples of human oral and dental biofilm and consequently if in the future oral isolates of this bacterium could be used for their probiotic/antibiotic activity in the treatment of oral infections sustained by susceptible pathogens. Since *B. bacteriovorus* can only be cultivated in the presence of a live bacterial pray and its concentrations in the environment are generally low, we decided to investigate its presence in oral samples by culture independent methods. To this purpose the polymerase chain reaction (PCR) method was used. PCR is a simple, fast, and reliable method, developed in 1983 by Nobel Prize Kary Mullis. It is widely used in molecular biology and diagnostics, to exponentially amplify species specific DNA sequences from mixed DNA samples, and relies on the ability of thermostable DNA polymerases, obtained from thermophilic bacteria, to specifically amplify a selected segment of DNA in the presence of short single-stranded oligonucleotides (primers) corresponding to the two extremities of the segment to be amplified.

Materials and methods

Patients. Twenty adult human subjects of both sexes were enrolled for this study. Inclusion criteria were age ≥18 years, presence of at least 20 natural teeth. Exclusion criteria were presence of removable prosthesis, presence of relevant systemic diseases (diabetes, immunopathologies, neoplasies, organ transplantations), use of immunomodulant or immunosuppressive drugs, pregnancy, hormonal treatments, assumption of antibiotics in the last 30 days.

Upon enrolment in the study patients were informed of the scopes of the study itself and were asked to sign an informed consent in accordance with criteria of the Helsinki Declaration of 1975, as revised in 2000.

This study was approved by the Ethical Committee of Policlinico Umberto I of Rome (Ref. 4790).

Microbial samples. Samples of oral and dental biofilm were obtained from each patient.

Samples of oral biofilm were obtained by sterile swabs streaked on the upper vestibular mucosa from the right first molar region to the left first molar region.

The swabs were then inserted in sterile tubes containing 1 ml of sterile, freshly prepared Reduced Transport Fluid (RTF),[28] and transported to laboratory for further processing.

Dental biofilm samples were obtained by streaking a sterile round-headed microbrush around the marginal region of a molar/premolar for each quadrant. Contamination by saliva was prevented by opportunely positioned cotton rolls. The terminal tip of each microbrush was then cut by sterile forceps, inserted in a 1.5 ml polypropylene microcentrifuge tube containing 0.5 ml of RTF, and transported to laboratory for further processing.

Processing of samples. Upon arrival to the laboratory microbiological samples were vortexed for 2 minutes to suspend bacterial cells; the suspension was then transferred to new sterile microcentrifuge tubes, sedimented by centrifugation at 10.000x g for 5 minutes and bacterial pellets were further processed for extraction of metagenomic DNA.

Extraction of metagenomic DNA was performed by the UltraClean® Microbial DNA Isolation Kit (Mo Bio – Qiagen Italia, Milan Italy) according to instructions of the manufacturer.

Concentration and purity of extracted DNA were assessed by UV spectrophotometry at 260 nm and 280 nm with a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany).

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Extracted metagenomic DNA samples were stored at −20°C until further analyses.

Molecular analyses. The metagenomic DNA extracted from each microbiological sample was used as the template for polymerase chain reactions (PCR) to detect specific sequences of the *B. bacteriovorus* genome. The Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen Life Technologies, Milan, Italy) chemistry was used for reactions.

PCR reactions were designed to amplify a 481 bp specific region of the *B. bacteriovorus 16SrRNA* gene using primers Bd529 F (5'-GGT AAG ACG AGG GAT CCT-3') and Bd1007 R (5'-TCT TCC AGT ACA TGT CAAG-3').[29] DNA primers were obtained from Invitrogen (Invitrogen Life Technologies, Milan, Italy).

PCR reactions were prepared in a total volume of 0.05 ml using 4 μl of each extracted metagenomic DNA as the template. Negative and positive control reactions were included for each set of reactions. Negative controls were prepared by inserting 4 μl of sterile molecular grade water instead of the metagenomic DNA sample. Positive control reactions were performed using 20 ng of genomic DNA extracted from *B. bacteriovorus* strain HD100 (Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSMZ, Braunschweig, Germany, strain DSM 50701) as the template.

PCR reactions were based on 35 cycles with denaturation at 96°C, annealing at 50°C, and extension at 72°C.

Amplification products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide. Electrophoresis chemicals were obtained from Merck Life Science (Milan, Italy). Reactions were considered as positive when an amplification product with motility comparable to the product obtained from positive control and corresponding to about 481 bp was observed. The 100 bp DNA Ladder (Invitrogen Life Technologies, Milan, Italy) was used as molecular size standard for electrophoresis.

All positive PCR products were purified from reaction mixtures using the PureLink™ PCR Purification Kit (Invitrogen Life Technologies, Milan, Italy) and sequenced using the BigDyeTM terminator chemistry (Applied Biosystems, Foster City, USA). The sequencing mixtures were analyzed on a DNA sequence analyser ABI3730 (Applied Biosystems, Foster City, USA). Sequencing was performed on both strands and sequences were analyzed with SEQ MATCH at the Ribosomal Database Project II website (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). The obtained sequences were also compared to gene sequences of known bacterial identities available in GenBank by means of the Basic Local Alignment Search Tool (BLAST) present at National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

Results

This study aimed to detect by molecular methods the presence of *B. bacteriovorus* in samples of oral and dental biofilm.

A total of 20 patients were selected and enrolled in the study (12 males and 8 females) ages ranging between 22 and 61 years (mean age 37.9).

Overall 20 samples of oral biofilm from the vestibular mucosa and 20 samples of dental biofilm from the region of the gingival margin were analyzed by PCR for the presence of *B. bacteriovorus*-specific DNA sequences (*16SrRNA* gene).

Analysis of PCR amplification products by 1.5% agarose gel electrophoresis evidenced the presence of amplification products with an apparent electrophoretic motility of 481 bp, congruent with the expected specific amplification product and corresponding to the amplification product obtained with positive control reactions, for 12 oral biofilm samples (60%) and for all 20 dental biofilm samples. In many cases intensity of amplification bands resulted very low, suggesting that concentration of the specific template in the sample was low.

Sequencing of amplification products retrieved sequences with 100% identity with the sequence of reference strain HD100 in 17 of 32 cases (53.1%), sequences with ≥99% identity in 11 cases (34.4%), and sequences with identities ranging between <99% and ≥97% in the remaining 4 cases (12.5%).

Discussion

To our knowledge this is the first survey specifically reporting the presence of *B. bacteriovorus* in human oral and dental biofilms. Studies performed in the last decade have already demonstrated that this predatory bacterium is part of the intestinal microbiome and that its abundance is directly related to intestinal health.[20]

The human body, and his digestive tract in particular, including the oral cavity, is inhabited by a complex array of microbial communities, characterized by high levels of complexity, whose composition is regulated by many different mechanisms.[1-6]

Recent improvements in molecular technologies for DNA sequence disclosure and analysis have enabled to investigate the relations existing between the composition of the microbiota at different sites of the human body, the underlying regulatory mechanisms, and their correlation with a surprising number of human diseases.[30]

Collected data have already enabled to develop new and innovative therapeutic approaches for different diseases, and promise to transform our approach to treat patients, reducing adverse events, and decreasing health care costs.[31]

Dysbiotic alterations of the oral microbiota were recognized as causative factors of periodontal disease and inflammatory peri-implant diseases.[32,33]

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Predation is certainly an important mechanism in natural environments to determine qualitative and quantitative equilibrium of resident microbiota and to allow the cycling of nutrients through the microbial loop.[34]

Direct evidence of the presence of a well-known predatory bacterium in the oral cavity strongly suggests that it participates in the complex interactions that help to determine qualitative and quantitative characteristics of biofilms in the different niches and are relevant for oral health and for the onset of different oral and systemic diseases.[3-9]

This was a preliminary pilot study aiming to evaluate if *B. bacteriovorus* is a member of the human oral and dental microbiota and consequently if in the future it could be used for its probiotic/antibiotic activity in the treatment of oral infections sustained by susceptible pathogens. To this purpose 20 samples of oral biofilm and 20 samples of dental biofilm from healthy subjects were analyzed. Analyses returned an overall positivity for the presence of *B. bacteriovorus*-specific DNA sequences of 32/40 (80%) and specificity of PCR amplification products was confirmed for a large majority of cases by sequence analysis. This study was not designed to evaluate the presence of *B. bacteriovorus* and its eventual correlation to oral diseases. Further studies are consequently necessary to evaluate the existence of any correlation between the abundance of *B. bacteriovorus* in biofilm samples and selected oral conditions including the presence/absence of periodontal disease and the conditions of peri-implant tissues.Previous studies reported that for almost all studied niches, including marine sediments, fresh water and animal reservoirs[20], the population of *B. bacteriovorus* is of low numeric consistency; although data presented here were not obtained by quantitative methods, the amounts of metagenome necessary to obtain positive PCR reactions in our hands suggest that also in the oral cavity *B. bacteriovorus* is represented in low numbers.

Several studies demonstrated that *B. bacteriovorus* is able to predate many relevant human pathogens,[35-37] including several important oral and periodontal pathogens[21-23] and that it does not exert toxic effects on cultured cells and in animal models.[38]

B. bacteriovorus was consequently proposed as an alternative/adjunctive therapeutic agent for different infections[24-26] including human periodontitis.[27]

The evidences presented in this paper that *B. bacteriovorus* is a member of the human oral microbiota strengthen any proposal of its use in the prevention/treatment of oral diseases associated with dysbiosis (periodontal diseases and periimplantitis) and of infections sustained by bacteria being susceptible to its predatory activity.

Future studies will be addressed at investigating the role of *B. bacteriovorus* in oral diseases and at selecting oral isolates of *B. bacteriovorus* and other predatory species to be used in the development of specific probiotic/antibiotic preparations that could prove useful preventive/therapeutic tools for oral dysbiosis and associated oral conditions.

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Conflicts of interest

The authors state that they have no conflict of interests with regards to this paper.

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