Urinary microbiota in patients with prostate cancer and benign prostatic hyperplasia

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Submitted: 6 May 2014
Accepted: 12 June 2014

Arch Med Sci 2015; 11, 2: 385–394
DOI: 10.5114/aoms.2015.50970

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Abstract

Introduction: Inflammation is associated with promotion of the initiation of various malignancies, partly due to bacterial infection-induced microenvironmental changes. However, the exact association between microbiota in urine, seminal fluid and the expressed prostatic secretions and benign prostatic hypertrophy and prostate cancer is not clear.

Material and methods: In the present study, we investigated the type of microbiota in the expressed prostatic secretions (EPS) of patients with prostate cancer and benign prostatic hyperplasia (BPH) by the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method using universal bacterial primers. In order to understand the possible association between various bacteria and prostate cancer, quantitative real-time PCR assay was performed to quantify the amount of strains of bacteria in urine, EPS and seminal fluid.

Results: The prostate cancer group had a significantly increased number of Bacteroidetes bacteria, Alphaproteobacteria, Firmicutes bacteria, Lachnospiraceae, *Propionicimonas*, *Sphingomonas*, and *Ochrobactrum*, and a decrease in *Eubacterium* and *Defluviicoccus* compared to the BPH group. The number of *Escherichia coli* in the prostate cancer group was significantly decreased in urine and increased in the EPS and seminal fluid, while the number of *Enterococcus* was significantly increased in the seminal fluid with little change in urine and EPS.

Conclusions: Based on these results, we suggest that there are significant changes in the microbial population in EPS, urine and seminal fluid of subjects with prostate cancer and BPH, indicating a possible role for these bacteria in these two conditions.

Key words: urinary microbiota structure, prostate cancer, polymerase chain reaction-denaturing gradient gel electrophoresis, quantitative real-time polymerase chain reaction.

Introduction

Prostate cancer is one of the most common cancers in men, especially in the elderly. Morbidity due to prostate adenocarcinoma increases to ~60% for men over the age of 60 years in the USA. It has long been suspected that chronic inflammation may have an important role in the initiation and progression of prostate cancer [1–3]. The observation that

Helicobacter pylori induces chronic inflammation and thus may pave the way to develop stomach cancer [3] lends support to such a belief that bacteria may also have a role in prostate cancer [3]. There is some support to this contention that prostatic inflammation may increase the risk of benign prostatic hyperplasia (BPH) and prostate cancer [4, 5].

It is difficult to detect many anaerobic bacteria present in various human body fluids, especially in the urine, expressed prostatic secretions (EPS) and seminal fluid, using traditional culture methods [6, 7]. Hence, in the present study, we employed culture-independent methods (polymerase chain reaction-denaturing gradient gel electrophoresis – PCR-DGGE) that are more reliable to detect various bacteria present in the human body fluids [8]. This method has previously been used by others to identify a variety of bacteria in various secretion of the human body [8–12].

Gram-negative enteric bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus* are frequently associated with urinary tract infections that may cause chronic bacterial prostatitis [13]. Furthermore, infections due to *E. coli* and *Enterococcus* are accompanied by elevated levels of pro-inflammatory cytokines that are related to cancer development and progression [14, 15]. Hence, it is reasonable to propose that chronic bacterial prostatitis induced by bacteria may enhance proinflammatory responses that may contribute to prostate cancer.

In order to verify this possibility, we investigated the bacterial population in the EPS of patients with BPH and prostate cancer by PCR-DGGE with 16S rDNA methods and quantified changes in the number of *E. coli* and *Enterococcus* by quantitative real-time PCR assay in the present study [16, 17].

Material and methods

Specimen collection

All male patients included in the study were under the age of 75 and diagnosed with BPH or prostate cancer. All patients were educated as to how to collect their urinary samples without contamination. The penis was cleaned with warm water and a 75%-alcohol tampon 3 times [18] and urine was collected in a 10-ml sterile tube by each study subject. Expressed prostatic secretions and seminal fluid were collected following prostatic massage in a 5.0-ml sterile tube for each subject. Urine, EPS and seminal fluid were carefully collected into the tubes without touching the interior wall of the sterile tube. Routine urine test was performed before sample storage. Urine, EPS and seminal fluid were all stored at –80°C for DNA extraction. The EPS samples from prostate cancer/BPH patients were pooled for the DGGE analysis.

DNA extraction

Samples were melted to liquid at room temperature (15–25°C) and stored on ice for DNA extraction. QIAamp DNA Mini Kit (Qiagen, USA) and distilled water were equilibrated to room temperature before DNA extraction. Twenty µl QIAGEN Protease was pipetted into the bottom of a 1.5 ml micro-centrifuge tube, and then a 200 µl sample (urine, EPS and seminal fluid) was added to the tube. Following this, 200 µl of Lysis buffer was added to the sample and mixed for 15 s, and the mixture was incubated at 56°C for 10 min in a water bath. Two hundred µl of absolute ethanol was pipetted into the mixture and mixed well. Following brief centrifugation, the mixture was carefully applied to a QIAamp Mini spin column without wetting the rim. On the other hand, following 1-min-long centrifugation at 8000 rpm, 50 µl of buffer AW1 was carefully added to the column without wetting the rim. An additional 1-min-long centrifugation at 8000 rpm was performed and the column was placed in a clean 2 ml collection tube. Five hundred µl buffer AW2 was added to the column and was centrifuged for 3 min at 14,000 rpm. Subsequently the column was kept in a clean 1.5 ml tube, 20 µl Elution buffer was added, and it was incubated at room temperature for 1 min. DNA was collected from the columns by centrifuging at 8000 rpm for 1 min. The DNA samples collected were subpackaged and stored at –20°C.

Polymerase chain reaction amplification

The concentrations of DNA in the samples were measured by ultraviolet spectrophotometer (Thermo Electron Corporation, USA). All DNA samples were amplified in the V3 regions of the 16S rRNA using universal bacterial primers (341F:5'-GTATTACCGCGGCTGCTGG-3'; 534R:5'-ACTCCTACGGGAGGCAGCAG-3') with a 40-bp GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') attached to the 5' ends of the reserve primers (534R) for DGGE analysis. The PCR mixture comprised 1 µl of Bestar Taq DNA Polymerase (2.5 U/µl, DBI Bioscience, Shanghai, China), 5 µl of deoxynucleoside triphosphates (dNTPs, 2 mM each), 5 µl of 10× Bestar Taq Buffer, 1 µl of each primer (10 µM) and 2 µl of extracted bacterial DNA (~60 ng). The total PCR reaction volume was 50 µl. The protocol for the PCR procedure was the touchdown protocol as described by Muyzer *et al.* [8] as follows: 94°C for 5 min; 20 cycles of touchdown PCR: denaturation at 95°C for 30 s, annealing at 65°C for 30 s with the temperature decreasing by

0.5°C/cycle until the annealing temperature was 55°C, and extension at 72°C for 30 s; 20 cycles of PCR: 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; final extension at 72°C for 5 min; 4°C hold. Polymerase chain reaction (PCR) was performed in a thermal cycler PCR system (BBI, Canada) and PCR products were checked by electrophoresis on 1.0% (w/v) agarose gel (Biowest Regular Agarose G-10, Spain). Electrophoresis was performed at a voltage of 100 V for 20 min with 1× TAE buffer, and visualized by ethidium bromide staining using a gel imaging system (Bio-Rad Laboratories Gel-Doc 2000, Oslo, Norway). All PCR products were stored at –20°C before DGGE electrophoresis.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis analysis was performed for the EPS specimens of prostate cancer and BPH, using the Bio-Rad Dcode mutation detection system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. DNA fragments with different sequences were separated in 8% polyacrylamide (acrylamide: bisacrylamide = 37.5 : 1; w/v) gels in 1× TAE buffer with 200 ng of each PCR product. A 30–60% denaturant gradient (100% denaturant was 7 mol/l urea and 40% (v/v) deionized formamide) was applied in the DGGE electrophoresis, which was performed in 1×TAE buffer at 180 V at 60°C for 4 h. Subsequently, the gels were washed with ultrapure water and stained with 5% Goldview dye for 30 min and photographed. DGGE graphs were digitized by Quantity One Analysis software (Gene Genius).

Denaturing gradient gel electrophoresis band sequencing and phylogenetic analysis

Denaturing gradient gel electrophoresis bands (#5, #6, #8, #10, #13, #15, #16, #17, #19) excised from the gel under UV and the DNA fragments were collected and purified with DNA Gel Extraction Kit (SK1135, Sangon, Shanghai, China) according to the manufacturer's instructions. The purified DNA was used as templates for PCR with the primers mentioned above. The PCR products were ligated into the pUCm–T vector (Sangon, Shanghai, China) and competent *E. coli* DH5α cells (Sangon) were transformed. The recombinant cells were selected and cultured overnight at 37°C. Subsequently, bacteria were collected, and DNA with the plasmid was extracted using the UNIQ-10 column kit (Sangon) following the kit instructions. Clones located in the same position as the original DGGE bands were sequenced (Sangon, Shanghai, China).

Similarity searches with obtained sequences were performed online based on the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) BLAST. We compared the 16S rDNA sequences of the selected bands and their identity was arrived at (by comparing with the NCBI GenBank database) when the similarity of the sequence was above 97%. Based on the similarity search results, a phylogenetic tree was constructed by the neighbor-joining method with the MEGA 5 software (version 5.05). The consistency of the relationship on the tree was assessed by bootstrap resampling. Bootstrapping was performed for 1000 trials in accordance with the Clustal X program [19].

qPCR assay for *Escherichia coli* and *Enterococcus*

Each DNA sample in this study was amplified with primers (VS8F: 5'-GGCGGATTAGACTTCGGCTA-3', VS9R:5'-CGTTTTGGCACTATTTGCCC-3') for *E. coli* and primers (Ent1F: 5'-TACTGACAAACCATTCATGATG-3', Ent2R:5'-AACTTCGTCACCAACGCGAAC-3') for *Enterococcus*. Each PCR reaction mix consisted of the following in a total volume of 20 µl: 10 µl of Bestar SybrGreen qPCR mastermix (DBI Bioscience, Shanghai, China), 0.5 µl of each PCR primer (10 µM), 2.5 µl of DNA template and 6.5 µl of distilled water. Polymerase chain reaction conditions employed were as follows: initial denaturation at 95°C for 2 min; 40 cycles for PCR reaction of 95°C for 10 s, 55°C for 30 s and 72°C for 30 s; melting curve at 65°C for 5 s and 60 cycles from 65°C to 95°C (10 s/cycle, 0.5°C/cycle).

A DNA sample with the maximum concentration was diluted 10, 10^2, 10^3, 10^4, 10^5 times and amplified under the same PCR conditions for the standard curves. All the samples were amplified in triplicate using Thermal Cycler (CFX-Touch, BIO RAD) and the Ct values were compared with the standard curve to obtain the relative quantity of *E. coli* and *Enterococcus*.

Statistical analysis

All results obtained were expressed in mean ± SD. Statistical analysis was performed by analysis of variance or by paired *t*-test when just two values were compared, using SPSS software version 9.1. Group differences were shown as * meaning $p < 0.05$, ** meaning $p < 0.01$, or *** meaning $p < 0.001$.

Results

Specimen characteristics

Specimens for the present study were obtained from patients with BPH and prostate cancer attending the Department of Urology, the First Affiliated Hospital of the College of Medicine, Zhejiang University. The mean age of the recruited subjects in both groups was 66.7 years. Urine and blood

Table I. Urine characteristics of patients with BPH

Patient no.	Occult blood	WBC [/µl]	Turbidity	Bacteria [/µl]
14	_	9.6	+	2.6
15	_	7.4	_	22.3
16	_	4.6	_	32.2
17	_	11.9	_	11.9
18	+	23.1	_	9.4
19	++	8.5	_	7.1
20	+++(OVER)	21.1	_	5.5
21	+++(OVER)	80.0	_	7.1
22	_	2.3	_	10.4
23	+	18.5	_	70.2
24	+	21.5	_	87.3
25	++	207.7	+	16.7
26	+	2.0	_	7.9
27	++	101.8	+	12.7
28	_	2.2	_	8.7
29	+++(OVER)	34.5	_	3.1
30	++	18.2	_	1.8
31	++	14.4	_	7.9
32	+	16.3	_	6.6
33	+	5.6	_	0.7
34	++	52.3	_	11.9

WBC – white blood cells.

Table II. Urine characteristics of patients with prostate cancer

Patient no.	Occult blood	WBC [/µl]	Turbidity	Bacteria [/µl]
1	+++(OVER)	444.9	+	22.3
2	+++(OVER)	131.1	+	68.3
3	+++(OVER)	157.4	_	5291.8
4	++	431.9	+	23.0
5	++	331.0	+	36.8
6	+++(OVER)	100.6	_	23.0
7	+++(OVER)	39.2	_	46.7
8	+++(OVER)	148.5	_	67.1
9	++	49.9	_	28.7
10	+++(OVER)	34.3	+	151.9
11	+	21.5	_	87.3
12	_	556.4	+	24602.5
13	+++(OVER)	30.9	_	39.1

Table III. Tumor markers of patients with prostate cancer

Patient no.	α-Fetoprotein [ng/ml]	CEA [ng/ml]	CA125 [U/ml]	Ferritin [ng/ml]	PSA [ng/ml]
1	1.8	1.6	7.8	146.0	0.624
2	3.8	2.4	19.4	441.0	5.447
3	6.1	5.4	6.8	59.4	12.117
4	2.2	1.2	20.9	6389.0	> 1000
5	1.6	2.3	212.2	457.4	140.866
6	5.1	4.9	26.9	399.7	37.694
7	3.1	4.8	15.1	382.8	17.790
8	2.1	1.4	6.6	221.7	28.892
9	3.3	4.1	12.5	308.2	16.599
10	3.7	4.7	9.8	171.5	2.936
11	2.3	2.6	8.3	153.9	6.342
12	2.7	3.2	4.2	156.3	11.900
13	2.9	4.3	8.9	151.3	1.917

CEA – carcino-embryonic, CA125 – carbohydrate antigen 125.

analysis was performed before specimen collection. Characteristics of the urine examination of the recruited patients are given Tables I and II. The characteristics of the tumor markers of patients with prostate cancer studied and their results are given in Table III. Compared to patients with prostatic hyperplasia, patients with prostate cancer had higher incidence of occult blood and turbidity, and a higher quantity of white blood count (WBC) and bacteria in the urine, suggesting that these patients might be having concurrent infection and inflammation that could be due to the presence of infection of the prostate or urethritis. Three inferences could be arrived at from these findings. They are: (i) active infection is present in the prostate at the time of the study; (ii) active infection is present in the urinary tract; and (iii) active infection is present in both the prostate and the urinary tract. Despite these possibilities and possible presence of active infection in the prostate and/or urinary tract, the results of the present study showed that there could occur a close relationship between infections and prostate cancer.

Polymerase chain reaction efficiency of 16s RNA

The total DNA of each specimen was extracted successfully and PCR assays for 16sRNA gene fragments of bacteria were performed using bacterial universal primers 341F and 534R. The amplified fragments of PCR were approximately 230 bp, as shown in Figures 1 and 2. Based on the results obtained, it is evident that 16s RNA gene fragments of bacteria in the specimens had good amplification efficiency.

Denaturing gradient gel electrophoresis analysis and sequencing results

Polymerase chain reaction-denaturing gradient gel electrophoresis fingerprinting analysis of patients with BPH and prostate cancer for predominant bacteria was used to identify EPS microbiota

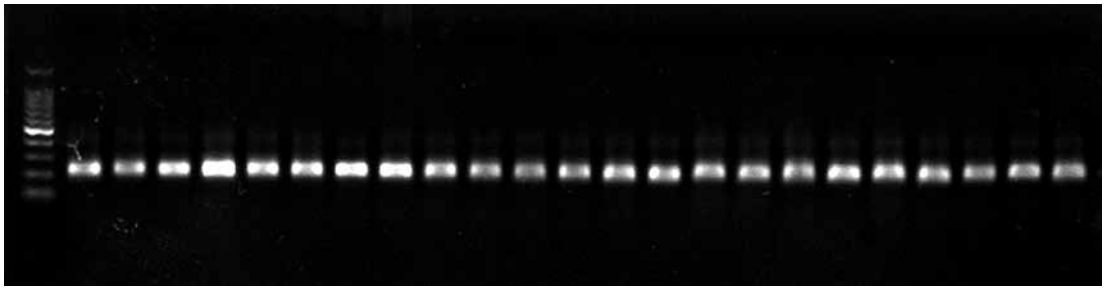

Figure 1. Gel profiles of the V3 region amplified by PCR from the extracted DNA from subjects with prostate cancer

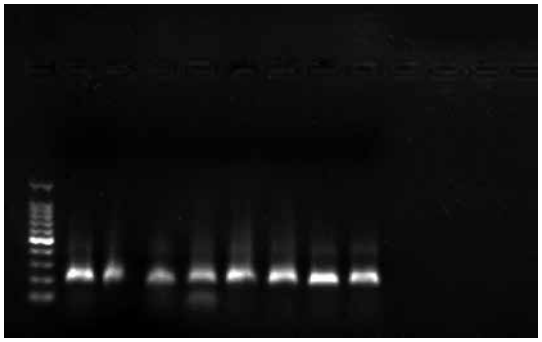

Figure 2. Gel profiles of the V3 region amplified by PCR from the extracted DNA from subjects with BPH

(Figure 3). The extracted DNA was amplified with universal primers targeting the hypervariable V3 region of the 16S rRNA gene. Based on PCR-DGGE fingerprinting analysis, the diversity of microbiota structures was presented with separated bands of different DNA sequences, as shown in Figure 3, which showed that there were about 20 discernible bands.

According to the DGGE profile of EPS, several bands of the prostate cancer group were discovered to be distinct from those present in the BPH group. These distinct bands, namely #5, #6, #8, #10, #13, #15, #16, #17 and #19, were identified as significant variables in discriminating the prostate cancer group from the BPH group. These distinct bands were sequenced to determine the exact microorganisms that were present in the urinary system during prostate cancer. The bands (#5, #6, #8, #13, #16, #17, and #19) that were brighter in the prostate cancer and bands (#10 and #15) in the BPH groups were amplified, and the lengths of these fragments were about 190 bp (Figure 4).

The sequences of prominent bands #5, #6, #8, #13, #16, #17, and #19 in the prostate cancer group matched with Bacteroidetes bacteria, Alphaproteobacteria, Firmicutes bacteria, Lachnospiraceae, *Propionicimonas*, *Sphingomonas*, *Ochrobactrum* and bands #10 and #15 of BPH matched with *Eubacterium* and *Defluviicoccus* species respectively. It is evident from these results that there were almost no significant differences in the diversity of bacteria between the groups except for the fact that seven kinds of microbes in the prostate cancer group were distinctly increased in quantity compared with those in the BPH group. However, *Eubacterium* and *Defluviicoccus* were present in lower amounts in the prostate cancer group compared with the BPH group.

Phylogenetic analysis

Sequences of the bands were blasted in the NCBI database and a sequence having above 97% similarity was regarded as the same. Every

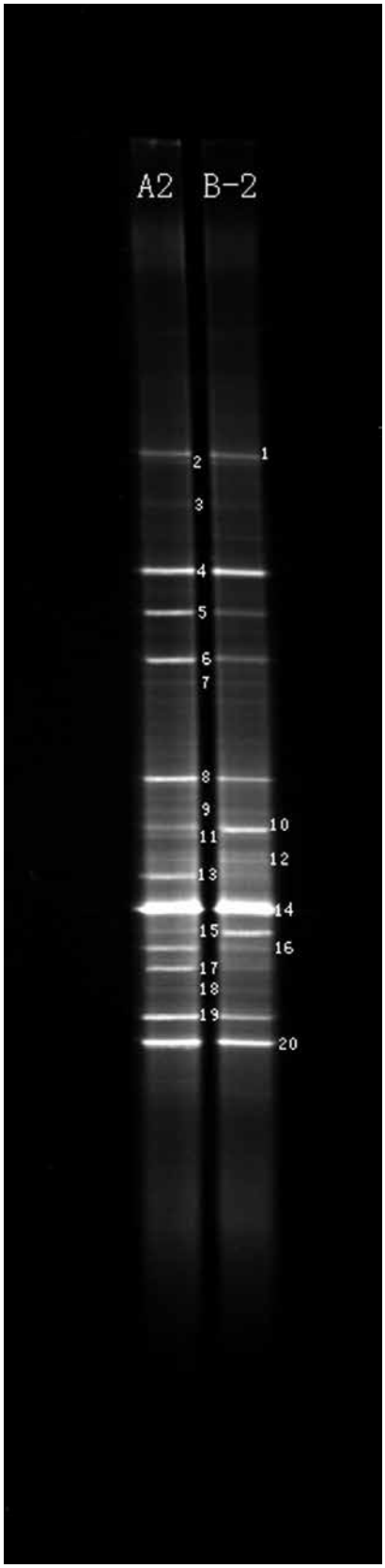

Figure 3. Gel profiles of the V3 region amplified by polymerase chain reaction from bands #5, #6, #8, #10, #13, #15, #16, #17 and #19. The lengths of fragments were approximately 190 bp (BPH (A2) and prostate cancer (B-2))

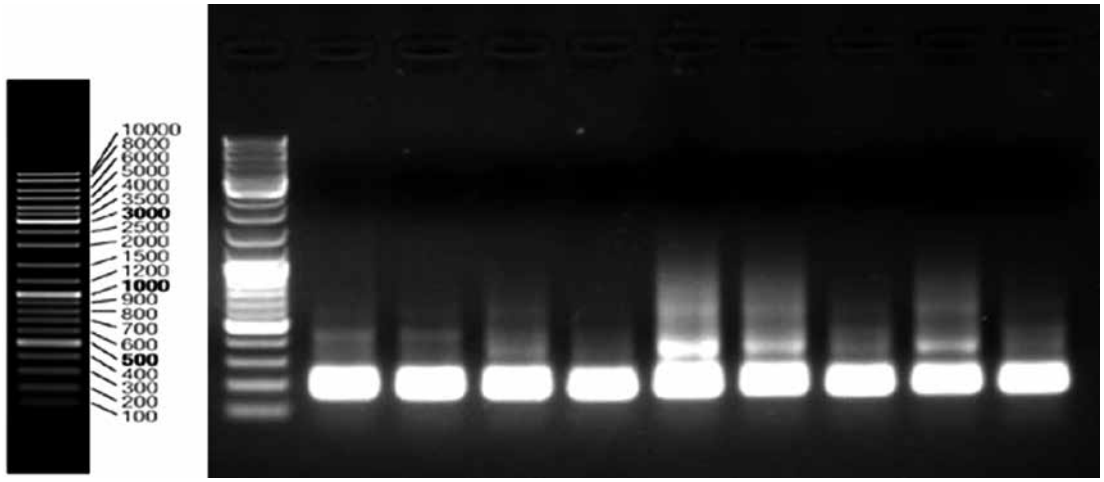

Figure 4. DGGE profile of EPS obtained from subjects with BPH (A2) and prostate cancer (B-2)

sequence of the band examined showed about 97% similarity. Among these strains, the one that showed the highest similarity of the same category was selected. Based on these results, the phylogenetic tree was created by MEGA 5.05 (Figure 5).

As shown in the phylogenetic tree (Figure 5), most of the strains were uncultured and values

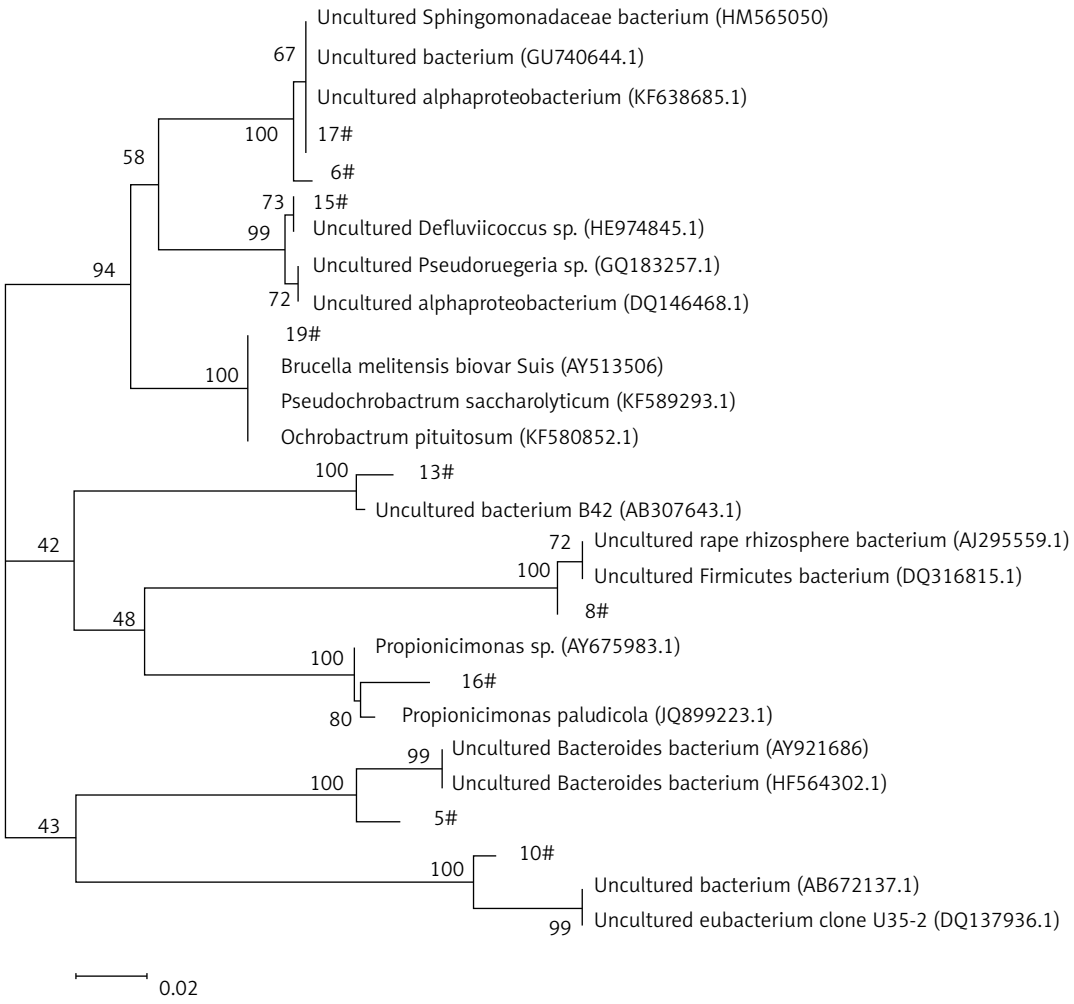

Figure 5. Phylogenetic tree of distinguished strains in prostate cancer group and BPH group

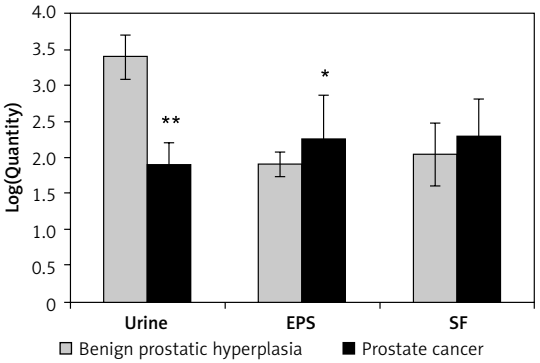

Figure 6. The qPCR results of *E. coli* in urine, EPS and seminal fluid (SF) obtained from subjects with BPH and prostate cancer

**p < 0.05, **p < 0.01; based on Students' t test.*

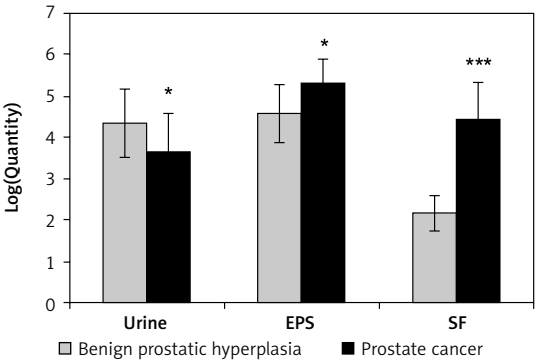

Figure 7. The qPCR results of *Enterococcus* in urine, EPS and seminal fluid (SF) obtained from subjects with BPH and prostate cancer

**p < 0.05, ***p < 0.01; based on Student's t test.*

on the branches of the tree showed a high confidence coefficient and close genetic relationships.

Quantitative polymerase chain reaction assays

The standard curves of both two PCR assays (*E. coli* and *Enterococcus*) showed a good linearity. The amount of *E. coli* in the prostate cancer group was statistically significantly decreased in urine and increased in EPS ($p < 0.05$), but showed no significant change in seminal fluid compared with the BPH group (Figure 6). The amount of *Enterococcus* in EPS in the prostate cancer group was significantly higher ($p < 0.05$) than that seen in the BPH group, while the amount of *Enterococcus* in urine of the prostate cancer group was significantly lower ($p < 0.05$) compared to that noted in the BPH group (Figure 7). In the seminal fluid, the quantity of *Enterococcus* in the prostate cancer group was found to be significantly higher ($p < 0.01$) compared to the BPH group (Figure 7).

Discussion

Previously, it was reported that prostatitis and prostatitis symptoms are associated with an increased risk of prostate cancer [5]. It is likely that bacteria might induce a chronic inflammatory state in the prostate that results in enhanced production of pro-inflammatory cytokines. Both neutrophils and macrophages may release proinflammatory molecules such as nitric oxide that have the propensity to cause genetic damage that could pave the way for enhanced cell proliferation and cancer. This is particularly interesting in the light of the reports that low-grade inflammation exists in cancer. Some studies did show that specific types of bacterial sequences could be present in prostatic tissue that may not be detected by traditional cell culture methods [20].

Molecular-based methods to identify and characterize microorganisms have contributed to microbial discovery. 16S rDNA-based PCR assays are more sensitive than the traditional methods that rely on microbial culture techniques [21, 22]. Some previous studies evaluated bacterial 16S rDNA sequences in prostatic tissue from patients with prostate cancer [23]. In the present study, using a combination of PCR-DGGE fingerprinting analysis and sequencing, combined with phylogenetic analysis, we observed that the microbiota present in the urinary system are significantly different between patients with BPH and prostate cancer.

Denaturing gradient gel electrophoresis fingerprints indicated that the urinary microbiota compositions in the EPS of prostate cancer were similar to those seen in BPH though there were significant differences in the quantities of specific strains between the groups. The sequencing results showed that EPS of prostate cancer patients were rich in Bacteroidetes bacteria, Alphaproteobacteria, Firmicutes bacteria, Lachnospiraceae, *Propionicimonas*, *Sphingomonas*, and *Ochrobactrum*, which might be involved in the progression of prostate cancer. Capsular polysaccharides produced by Bacteroidetes bacteria are important pathogenic factors that can cause suppurative lesions in the abdominal cavity and other organs. *Sphingomonas* is a genus of Gram-negative bacteria that belongs to the Alphaproteobacteria, which is one of the commonest groups associated with urinary tract infection. Hence, increase of Alphaproteobacteria (band #6 and #17) in the prostate cancer group may be relevant to the infection and inflammation of the prostate seen in patients with prostate cancer. Firmicutes bacteria account for the largest proportion of the human gut microbiome and are known to be involved in energy resorption and obesity [24]. The number of Firmicutes in vertebrates including humans is considered to be diet-dependent and correlates

with caloric intake. Since a high-fat and high-caloric diet is widely believed to be an important factor involved in the genesis of prostate cancer, it is reasonable to propose that the increased number of Firmicutes (band #8 and #13) seen in the EPS of patients with prostate cancer may have a role in its pathogenesis. *Propionicimonas* belongs to the Propionibacterineae, which are the main anaerobic bacteria in EPS of prostatitis patients. *Ochrobactrum* is an opportunistic pathogen. In the present study, we observed that the quantity of *Ochrobactrum* in the EPS of prostate cancer patients was much higher compared with BPH, which suggests the immune dysfunction of these patients. This implies that efforts to suppress the growth of *Ochrobactrum* could be of significant benefit in the prevention of prostate cancer development.

The results of the present investigation showed that *E. coli* and *Enterococcus* are present in significantly large number in the EPS and seminar fluid of subjects with prostate cancer compared to BPH. Increased levels of these two types of bacteria in EPS and seminal fluid indicate that a significant degree of inflammation occurs in those with prostate cancer.

Several previous studies have used prostate cancer tissue to detect the presence and influence of bacteria in prostate cancer [23, 25, 26]. In contrast, we collected EPS to investigate the presence and possible shift in the type and quantity of microbiota in BPH and prostate cancer. Expressed prostatic secretions are secreted by the prostate and pass through the urethra, which is connected to the skin. Hence, it is likely that bacteria in the male reproductive tract and urethra could access EPS, and so their analysis could more likely reflect a shift in the type of bacteria present in the prostate with different diseases.

In conclusion, bacterial flora in EPS of subjects with prostate cancer is different from that seen in BPH. These results suggest that ecological dysbiosis of the bacterial community in the EPS might play an important role in the pathobiology of prostate cancer.

Conflict of interest

The authors declare no conflict of interest.

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