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ORIGINAL ARTICLE

Coryneform bacteria in human semen: inter-assay variability in species composition detection and biofilm production ability

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Background: Coryneform bacteria constitute an important segment of male urogenital microbiota. They have been generally considered as saprophytes, although some species have been associated with prostatitis as well. At the same time, biofilm infections have been suspected as a cause of prostatitis.
Objective: To identify a set of coryneform bacteria isolated from semen of either healthy men or prostatitis patients applying different methods to reveal inter-assay variability and to determine their ability of adhesion and biofilm production.
Design: Coryneform bacteria were identified by API Coryne 2.0 biochemical identification system and 16S rDNA sequencing using different primer sets. Quantitative assessment of biofilm production was performed using crystal violet binding assay method.
Results: The most common species were *Corynebacterium seminale*, *C. minutissimum*, and *Dermabacter hominis*. Altogether 14 species and related genera were found. We observed the best inter-assay agreement when identifying *C. seminale*. Biofilm was observed in 7 out of 24 strains. The biofilm-producing strains belonged to *Arthrobacter cumminsii*, *Dermabacter hominis*, *C. minutissimum*, and *Actinomyces neuii*. No differences were found between the strains originating from prostatitis patients and healthy men. *Dermabacter hominis* strains were more potent biofilm producers than *C. seminale* strains ($p = 0.048$).
Conclusions: We can conclude that a wide variety of coryneform bacteria can be found from the male genital tract, although their exact identification is problematic due to insufficient representation in databases. Nearly one third of the strains are able to form biofilm that may give them an advantage for surviving several host- and treatment-related conditions.

Keywords: *Corynebacterium*; *coryneform bacteria*; *identification*; *biofilm*; *prostatitis*; *semen*

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The healthy upper male genital tract is usually sterile and void of microorganisms, while the urethra contains normal microbiota. At the same time, cultures of male accessory gland secretions (semen, expressed prostatic secretion, post-massage urine) in case of prostatitis frequently reveal abundant microbial communities containing both opportunistic pathogens and the bacteria that are generally considered as commensal (or saprophytic) in nature (1–5).

One set of those commensals often isolated from the semen of both healthy men and prostatitis patients are coryneform (*koryne* – club, *forma* – shape in Greek) species (6–10). They form an important segment of male urogenital microbiota. Coryneform bacteria are pleomorphic gram-positive rods, sporeless, without capsule, and often appear in gram slide as hieroglyph-like clusters. *Corynebacterium diphtheriae* is highly relevant for medicine while *Corynebacterium glutamicum* is of major interest to biotechnology. Other coryneform species have gained less attention. In association with prostatitis, these have been generally considered as saprophytes, although at least in one instance, unknown *Corynebacterium* strains seemed to associate with prostatitis, and their apparent disappearance in the course of therapy indicated their possible relevance for prostatitis (9). Several other coryneform species have emerged as opportunistic

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pathogens while some may provide protection from invading pathogens (6, 7, 9, 10).

Biofilm infections have been suspected as a cause of prostatitis for some time. Biofilms and adhesion have been scrutinized in association with prostatitis in animal models and clinical materials since at least 40 years ago (reviewed by 10). Curiously, prostatic calcifications associate positively with infection and negatively with pelvic floor spasm, as if suggesting a distinction between a prostatitis caused by calcificate-associated biofilm infection and prostatitis caused by a non-infectious mechanism (11). It has been shown by electron microscopy and culture that prostate calcifications may provide a surface for biofilm (12) but respective information on corynebacteria is not available.

The aim of this study was to identify a set of coryneform bacteria – those isolated from semen of either healthy men or prostatitis patients – with different genomic and biochemical methods, and to determine their *in vitro* ability of adhesion and biofilm production.

Materials and methods

Origin and identification of strains

Twenty-four coryneform strains were obtained from Human Microbiota Biobank (HUMB, http://biomedicum.ut.ee/armb/biobank/) of Tartu. The strains were originally isolated from either healthy volunteers ($n=7$), men with inflammatory prostatitis ($n=12$), or otherwise healthy men who were male partners of the couples with idiopathic infertility ($n=5$). Because the latter were without prostatitis (including asymptomatic inflammation, etc.) they were grouped together with healthy volunteers. We included antibiotic use within the past 3 months as one of several exclusion criteria.

Detailed information about study group and sample collection procedure is given elsewhere (8). Briefly, the samples were cultured quantitatively to detect anaerobic, microaerophilic and aerobic bacteria within 1 h from collection. Aerobic (blood agar) and microaerobic (MRS agar and *Gardnerella vaginalis*-selective agar in 10% CO_2 atmosphere) cultures were incubated at 37°C for 1–3 days, anaerobic cultures (Wilkins–Chalgren media in an anaerobic glove box) for 3–5 days. Colonies with different morphology were Gram stained and examined microscopically. The strains in HUMB had been characterized by β-glucuronidase test or, if negative, by API Coryne 2.0 biochemical identification system (bioMérieux, France) according to manufacturer's instructions (the pre-sequencing identification of the strains is shown in Table 1).

Finally, the strains were identified by sequencing method. We chose two primer sets for identification of coryneform bacteria: broad-range primers (5'-GTTAAGTCCCGCAAGGA-3' and 5'-AGGAGGTGATCCAGCC-3') for 440 bp 16S rRNA product and latter primers (5'-CGTAGGGTGCGAGCGTTGTCCG-3' and 5'-CGTTGCGGGACTTAACCCAACATCTC-3') for 516 bp 16S rRNA product according to Adderson et al. (13). Sequencing was performed with Sequenase sequencing kit technology from Amersham, Slough, UK. The results were analyzed with BLAST (NIH GenBank) software.

Biofilm assay

Quantitative assessment of biofilm production was performed using crystal violet binding assay method modified from the classic Christensen microwell assay (14). Briefly, strains were grown in Mueller-Hinton broth for 48 h in ELISA plate together with negative control; growth was verified by measuring optical density (OD) with ELISA plate reader. Then, the broth was discarded, and the wells were washed and then stained with crystal violet. Note that crystal violet stains the cells rather than slime. The analyses were performed in quintuplicates and the median value was used for analysis. Six wells were used for negative control. Extinction at 490 nm of the inoculated wells was measured with an ELISA plate reader after 48 h growth. After 48 h incubation, the broth was removed, the plates were rinsed and the wells were stained with crystal violet. After staining and drying, the wells were read again with ELISA plate reader with measuring the extinction at 620 nm. Medians of quintuplicates were used for calculations. Arbitrarily, we considered the strains as biofilm producers if their extinction at 620 nm after staining with crystal violet was at least twice the mean of negative controls.

Statistical analysis

For statistical analyses, SigmaStat (Systat Software, Chicago, IL) and Excel (Microsoft, Redmond, WA) software programs were used. The differences between the groups were calculated with Fisher exact test, *t*-test (in case of normal distribution), and Mann–Whitney rank sum test (in case of nonparametric distribution). Statistical significance was assumed at $p<-0.05$ for all parameters.

Results

Altogether, 14 different coryneform bacteria were found from the investigated semen samples (Table 1). The most common species in semen were *C. seminale*, or as it is synonymously called, *C. glucuronolyticum* ($n=5$), *C. minutissimum* ($n=4$), and *Dermabacter hominis* ($n=4$) as identified by sequencing methods. No statistically significant differences between the subject groups were observed.

The distribution of species was heterogenous, and the sequencing method sometimes produced results that conflicted with another sequencing method (exact match between 16S and latter primer identification was seen in

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Table 1. Identification of the coryneform strains

Strain	16S rRNA ID	%	Latter primer ID	%	API Coryne	ID that was used for biofilm analysis
CM7	*Actinomyces neuii subs. neuii*	*100*	*Actinomyces neuii subs. anitratus*	100	Not analyzed	*Actinomyces neuii*
CM2	*Arthrobacter cumminsii*	*100*	*Arthrobacter cumminsii*	*99*	*Cellulomonas/Microbacterium*	*Arthrobacter cumminsii*
10040G	*Arthrobacter sp*	*96*	*Dermabacter hominis*	*100*	*Cellulomonas/Microbacterium*	*Dermabacter hominis*
10040R1	*Arthrobacter sp.*	*97*	*Dermabacter hominis*	*99*	*C. seminale*	*Dermabacter hominis*
03019A	*Brevibacterium sp.*	*97*	*Brevibacterium lutescens*	*100*	*Corynebacterium sp.*	*Brevibacterium lutescens*
04016D	*Brevibacterium sp.*	*98*	Uncultured bacterium *clone ncd2467c01c1*	*99*	*C. propinquum*	*Brevibacterium sp.*
01007A	*C. aurimucosum/nigricans*	*98*	Uncultured bacterium	*100*	*Corynebacterium* group G	*C. nigricans/aurimucosum*
SP9F	*C. aurimucosum*	*99*	Uncultured bacterium	*99*	*Corynebacterium* group G	*C. aurimucosum*
CM12-1	*C. freneyi*	*96*	*C. freneyi*	*99*	*C. striatum/amycolatum*	*C. freneyi*
02005P1	*C. seminale*[$]	*99*	*C. seminale*	*99*	*C. seminale*	*C. seminale*
03016B2	*C. seminale*	*99*	*C. seminale*	*100*	*C. seminale*	*C. seminale*
03026B	*C. seminale*	*99*	*C. seminale*	*100*	*C. seminale*	*C. seminale*
SP12B2	*C. seminale*	*99*	*C. seminale*	*96*	*C. seminale*	*C. seminale*
CM12-4	*C. seminale*	*100*	*C. seminale*	99	not analyzed	*C. seminale*
02016C	*C. glutamicum*	*91*	Uncultured bacterium	100	Could not be identified	*C. glutamicum*[€]
CM21	*C. hansenii*	*96*	*C. amycolatum*	99	Not analyzed	*C. amycolatum*
01007Q	*C. minutissimum*	*98*	Uncultured bacterium	100	Could not be identified	*C. minutissimum*
SP16B	*C. minutissimum*	*89*	*C. minutissimum*	*98*	*Corynebacterium* group G	*C. minutissimum*
CM17	*C. minutissimum*	*98*	Uncultured bacterium	99	Not analyzed	*C. minutissimum*
CM18	*C. minutissimum*	*98*	Uncultured bacterium	100	Not analyzed	*C. minutissimum*
CM14	*C. ureicelerivorans*	*93*	Uncultured bacterium	100	Not analyzed	*C. ureicelerivorans*[€]
SP41C	*Dermabacter hominis*	*100*	*Dermabacter hominis*	*99*	*Dermabacter hominis*	*Dermabacter hominis*
12034F1	*Dermabacter hominis*	*99*	*Dermabacter hominis*	*99*	*Dermabacter hominis*	*Dermabacter hominis*
SP6C	*Zimmermannella alba/ Pseudoclavibacter sp.*	*97*	*Agrococcus terreus/ Leifsonia naganoensis*	*99*	*C. afermentans/coyleae*	*Zimmermannella alba/ Pseudoclavibacter sp./ Agrococcus terreus/ Leifsonia naganoensis*[¥]

[$]Synonym of *Corynebacterium glucuronolyticum*.
[€]The estimated likelihood that this species is identified correctly is less than 96%. Interpret with caution.
[¥]A coryneform strain with identification signature that discourages suggesting a particular species.

11 out of 24 strains), or both of them produced results that did not allow any identification at all. Therefore, the identification was not conclusive for many of the strains and their identification can be considered presumptive. We observed the best inter-assay agreement when identifying *C. seminale*. API results were in clear agreement with genomic methods in 6 of 18 cases (4 *C. seminale* and 2 *Dermabacter hominis* strains).

Biofilm was observed in 7 out of 24 strains, assuming that biofilm yields OD greater than two (0.123) or four (0.246) times greater than that of negative control, respectively (Table 2). The biofilm-producing strains were *Arthrobacter cumminsii* (from prostatitis patient), *Dermabacter hominis* (two strains from patients, one from a healthy man), *C. minutissimum* (one strain from a patient, one strain from a control), and *Actinomyces neuii* (isolated from a healthy man). No differences were found between the strains originating from prostatitis patients and healthy men. *Dermabacter hominis* strains were more potent biofilm producers than *C. seminale* strains ($p = 0.048$).

Discussion

We investigated 24 coryneform strains originating from the male genital tract and could identify 21 of them to species level with at least 96% confidence. The most frequent species were *C. seminale*, *C. minutissimum*, and *Dermabacter hominis*. Seven strains were biofilm producers.

Our main interest was related to etiopathogenesis of chronic prostatitis (CP), therefore, half of the strains originated from CP patients. The causes of this widespread malady are largely unknown. Patients with CP/Chronic Pelvic Pain Syndrome (CPPS) (category NIH III

Table 2. Biofilm production of coryneform bacteria

Species	Number of strains	Biofilm OD (extinction at 630 nm)		Number of biofilm producers
		Range	Mean ± SD	
Arthrobacter cumminsii	1	0.327	0.327	1
Actinomyces neuii	1	0.296	0.296	1
Brevibacterium lutescens	2	0.063–0.299	0.181 ± 0.167	1
C. amycolatum	1	0.093	0.093	0
C. aurimucosum	1	0.065	0.065	0
C. freneyi	1	0.113	0.113	0
C. seminale[$]	5	0.057–0.091	0.070 ± 0.014	0
C. minutissimum	4	0.066–0.247	0.120 ± 0.085	1
C. nigricans/aurimucosum	1	0.072	0.072	0
C. ureicelerivorans	1	0.072	0.072	0
Dermabacter hominis	4	0.085–0.482	0.300 ± 0.170	3
Zimmermannella alba/Pseudoclavibacter sp./ Agrococcus terreus/Leifsonia naganoensis[¥]	1	0.063	0.063	0
C. glutamicum[€]	1	0.069	0.069	0
Subject group	Number of strains	Range	Mean ± SD	Number of biofilm producers
Healthy	12	0.057–0.299	0.142 ± 0.100	3
Prostatitis	12	0.063–0.482	0.154 ± 0.150	4

[$]Synonym of *C. glucuronolyticum*.
[¥]A coryneform strain with identification signature that discourages suggesting a particular species.
[€]The estimated likelihood that this species is identified correctly is less than 96%. Interpret with caution.

prostatitis) or asymptomatic inflammatory prostatitis (category NIH IV prostatitis) usually lack 'traditional' uropathogens in prostate-specific materials. To this date, no single etiopathogenetic mechanism has been proved yet, but an interrelated and multifactorial cascade has been proposed (15) where an initiating event (infection, trauma, etc.) may lead to immunologic stimulation, inflammation, neurogenic stimulation, neuropathic damage with afferent nerve upregulation, and ultimately, pain. Whether and when the usual commensals of male genital tract may act as pathogens is a good question without a good answer. Hua et al. (16) have reviewed the topic of unusual pathogens and concluded that specific microorganisms (atypical pathogens) explain the 'unexplained' prostatitis in up to a 10th of patients. Studies have also suggested the role of immune cross-reactivity (17) and association with urinary tract infection history (18).

Former studies by our research group had revealed numerous different aerobic, anaerobic, and microaerophilic microorganisms in the seminal fluid (1, 8, 19, 20) that coincided with the data of Szöke et al. (21). The main difference between healthy controls and inflammatory prostatitis patients was quantitative – the latter harbored significantly higher total concentration of bacteria (mean 10^5 CFU/ml) as well as a higher number of different species (mean 5) in their semen than controls (mean 10^3 CFU/ml and 3 species). This finding was somewhat surprising given that we were expecting monoinfection. Instead, we found abundant polymicrobial communities that may be comparable to bacterial vaginosis in women, suggesting that prostatitis might be viewed as an unfavorable shift in the balance of genital tract microbiota. Our finding fits to the hypothesis of Liu et al. (22) that dysbacteriosis in the male genital tract may be an underlying, primary cause of CP and that the wide use of antibiotics may be an initiating risk factor for prostatitis.

During our former studies (1, 8, 19) we found that coryneform bacteria constitute a significant part of these microbial communities, being present in three fourths of men. Earlier nucleotide-based studies by Tanner et al. (9) and Lee et al. (23) have also shown that *Corynebacterium sp.* were the most common bacteria in the prostatitis patients' expressed prostatic secretion or urine: they were more prevalent, abundant, and represented by higher number of species in prostatitis patients than in controls. Tanner et al. (9) even found some unidentified species similar to *Corynebacterium coyleae*, *Corynebacterium imitans*, and *C. seminale* that were restricted to the prostatitis patients, exclusively.

In the present study, we applied molecular identification for the seminal strains collected during our previous studies and tested their biofilm formation ability as putative virulence factor. For identification of the strains, we used two primer sets (13). The broad, less-specific 16S primer yielded more results with a species name than the

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latter primer. The latter primer often failed to provide a name for a particular strain, but if it did, then the identification was usually with higher confidence than in the case of 16S primer. The study resulted in 14 species and related genera. The most common species in semen were *C. seminale, C. minutissimum,* and *Dermabacter hominis*. No statistically significant differences between the subject groups were observed, which may be partly associated with the low number of strains. As concerns specific bits of information regarding particular species, *C. seminale* was first isolated from the semen of a prostatitis patient (6), although it can be found frequently in the semen of healthy men, as was confirmed by our study. *C. minutissimum* is a coryneform bacterium associated mainly with erythrasma, but more dangerous opportunistic infection cases also have been recorded (24, 25). That species has been isolated from prostate secretion as well (10). One strain of our study gave conflicting BLAST results (*Zimmermannella*, *Leifsonia*, *Agrococcus*, or *Pseudoclavibacter*), although all these bacteria are coryneform bacteria. None of them has been associated with the male genital tract before. *Brevibacterium* and *Dermabacter* species have been found from the skin of healthy adults, and seldom from various clinical samples (26). *Actinomyces neuii* is an atypical actinomycete (27) that has been associated with opportunistic infections.

The majority of the strains were previously identified by a biochemical method, Coryne API (bioMerieux), which had poor agreement with previously mentioned genomic methods. Although in earlier studies Coryne API has been lauded as a valuable species identification tool, in our study the concordance with genomic methods was not encouraging. At the same time, biochemical signatures may still give some additional information that could be related to disease (28). *Corynebacterium* group G (according to API profile) strains that were associated with prostatitis were identified as *C. minutissimum*, *C. aurimucosum*, and *C. aurimucosum*/*C. nigricans* with sequencing-based methods. During biochemical identification these strains did not match the *Corynebacterium* group G description perfectly. (They had large rather than tiny colonies, and these strains did not ferment fructose.) *Corynebacterium* group G is a heterogenous group and it may contain several unidentified *Corynebacterium* species. For example, *C. pilbarense* was identified from this group rather recently (29).

It had been also speculated (but not proven) that coryneforms could grow in the prostate as a biofilm that would enhance their antibiotic resistance (9) as uropathogenic coli bacteria do (12). The respective data regarding minimal bactericidal concentration (MBC) of vancomycin, moxifloxacin, or ciprofloxacin for *C. urealyticum* biofilm is about two or three orders of magnitude greater than the MBC of respective planctonic forms (30). Therefore, we aimed to find out whether coryneform bacteria have a proclivity to produce biofilm *in vitro*. According to our data, biofilm production was characteristic of 29% of the strains belonging to different species and originating from different patients' groups. It was interesting to note that biofilm producers and non-producers formed two distinct populations as if there were a binomial distribution of biofilm production. Some former studies have shown biofilm production ability of corynebacteria and these bacteria have been associated with catheter and prostheses infections, too (31–34). Hence, we might classify coryneform bacteria as possible or probable prostate pathogens (15), given that their initiatory, propagatory, or complicatory role remains yet unproven. Microbiologically, they may be responsible for recurrence of infections that render antibiotic therapy ineffective (35); clinically, the initiatory role may be masked by symptom-propagating neural sensitization (15). Complicatory role of bacterial overgrowth may seem likely as well (22).

In summary, a wide variety of coryneform bacteria can be found from the male genital tract, although their exact identification is problematic due to insufficient representation in databases. Nearly one third of the strains are able to form biofilm that may give them an advantage for surviving several host- and treatment-related conditions.

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