

SHORT COMMUNICATION

Coryneform bacteria in semen of chronic prostatitis patients

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Summary

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Our aim was to compare the presence and species composition of coryneform bacteria in chronic prostatitis patients and controls. Semen of 50 men with inflammatory prostatitis and 59 controls (without pelvic pain/discomfort complaints and leukocytospermia) was investigated. First-catch urine was additionally investigated in 36 men (30 with and 6 without prostatitis). Coryneform bacteria were found in semen of 76% men with inflammatory prostatitis and 83% controls. More than half of the isolates were identified as *Corynebacterium seminale*. Prostatitis patients with severe leukocytospermia (>1 million white blood cells per ml) harboured significantly more *Corynebacterium* group G (33% vs. 2%, $p = 0.0003$) and *Arthrobacter* sp. (17% vs. 2%, $p = 0.03$) in comparison with controls. Nine species of coryneforms with high concentration ($\geq 10\,000$ CFU per ml) were found in prostatitis patients as against only four species in controls. Half of the men harboured corynebacteria in semen as well as in urine, 22% of men in semen only, and 3% in urine only. The total concentration of coryneforms was greater in semen than in urine (median 5000 vs. 100 CFU per ml, $p = 0.053$). We suggest that although coryneforms are generally considered as saprophytes, they are not uniform and some species (*Corynebacterium* group G and *Arthrobacter* sp.) may be associated with inflammatory prostatitis.

Introduction

Chronic prostatitis is a common affliction, affecting men of all ages. Studies suggest that 2–14% of adult men have symptoms of prostatitis at any given time (Mehik *et al.*, 2000; Krieger, 2004), and up to 50% of men suffer from prostatitis at some point during their lifetime (Stamey, 1980). The etiopathogenesis of prostatitis is not adequately understood. Central to the controversy is whether the underlying cause is bacterial, autoimmune, or a primary neuromuscular pain syndrome. Routine aerobic cultures of prostate-specific specimens show known uropathogens in fewer than 10% of patients. Moreover, bacterial and leukocyte counts hardly correlate with severity of symptoms (Tanner *et al.*, 1999; Schaeffer *et al.*, 2002).

At the same time, even routine cultures of the semen or expressed prostatic secretions of chronic prostatitis patients frequently reveal abundant microbial communities containing the bacteria termed generally as sapro-

phytes. One of the commonest isolates are coryneform bacteria (Rehewy *et al.*, 1979; Willen *et al.*, 1996; Devriese *et al.*, 2000), including *Corynebacterium seminale* that was discovered first from the male urogenital tract (Riegel *et al.*, 1995) and also several difficult-to-culture and unculturable coryneforms (Domingue *et al.*, 1997; Tanner *et al.*, 1999). Some studies have indicated the relatedness of these microorganisms to prostatitis (Drach, 1974; Domingue & Hellstrom, 1998; Tanner *et al.*, 1999).

Coryneform bacteria are gram-positive irregular rods that are ubiquitous in nature. 'Coryneform' is an arbitrary term but it is generally accepted that a species is coryneform when it is facultatively anaerobic, does not form spores and can likely be observed on gram-stained slide as Chinese letters composed of gram-positive irregular rods, possibly club-shaped ('coryne' is Greek for 'club'). The largest genus in this group *Corynebacterium* was created in 1896 to accommodate the causative agent of diphtheria *C. diphtheriae*. Nearly 60 species of mostly human and animal origin have been added later.

Chemotaxonomically, this genus includes species that possess wall chemotype IV, short-chain mycolic acids, and DNA G+C contents ranging from 51 to 63 mol%. During the past decades the taxonomy of the genus *Corynebacterium* has undergone dramatic changes because more advanced identification methods have become available. Both the re-evaluation of existing data and detection of new species have subjected the genus *Corynebacterium* to many changes. The narrower definition of this genus has resulted in the transfer of several species to other genera such as *Arthrobacter*, *Bogoriella*, *Cellulomonas*, *Dermabacter*, *Rhodococcus*, and many others, forming a heterogeneous group of morphologically related genera (Funke *et al.*, 1997; Khamis *et al.*, 2005). Although coryneform bacteria are usually considered as part of the normal human flora or environmental contaminants, they have increasingly been recognized as a cause of life-threatening diseases (Bernasconi *et al.*, 2004).

As knowledge about male genital tract coryneforms is scarce, we aimed to compare their presence and species composition in the seminal fluid of chronic prostatitis patients and controls.

Materials and methods

Study group

The study was carried out between September 2003 and May 2005 at Tartu University Hospital. The study group included 109 men, of which 37 men were the participants of the prospective study of the etiopathogenesis of chronic prostatitis, and 72 men were the participants of the

prospective study Environment and Reproductive Health (EU 6th FP project QLRT-2001-02911).

In 50 out of 109 men, leukocytospermia (>0.2 million WBC per ml of semen) was found (Punab *et al.*, 2003); therefore, NIH IIIa category prostatitis (20 men) or NIH IV category prostatitis (30 men) was diagnosed in them according to the NIH Classification of the Prostatitis Syndromes (Krieger *et al.*, 1999; Table 1). Of these 50 men, 18 had severe inflammation (>1 million WBC per ml) and 32 had moderate inflammation (0.2–1 million WBC per ml). The remaining 59 men had neither pelvic pain/discomfort complaints nor leukocytospermia; therefore, they served as a control group (Table 2). The mean age of prostatitis patients was 28.5 (SE \pm 0.62) years and 20.0 (\pm 1.32) years in controls.

All subjects were at least 18 years old. Exclusion criteria were stated according to the suggestions of the NIH workshop on chronic prostatitis (National Institutes of Health Summary Statement, 1995). None of the men had received antimicrobial therapy within 3 months. All men gave consent for the additional microbiologic studies of their semen.

Samples

Semen samples were collected by patients after they washed their glans penis with regular soap and water, and urinated. The samples were obtained by masturbation and were collected in a sterile collection tube. After ejaculation, the semen was incubated at 37 °C for 25–45 min for liquefaction. The first-catch urine was additionally

Table 1 National Institutes of Health classification of the prostatitis syndromes

| Category | Type | Description | Presentation |
|----------|--|--|--|
| I | Acute bacterial prostatitis | Acute infection of the prostate gland | Acute febrile illness associated with perineal and suprapubic pain, dysuria, and obstructive voiding symptoms |
| II | Chronic bacterial prostatitis | Chronic infection of the prostate gland | Recurrent urinary tract infections with pain and voiding disturbances |
| III | Chronic prostatitis/chronic pelvic pain syndrome | Chronic genitourinary pain in the absence of uropathogenic bacteria localized to the prostate gland employing standard methodology | Chronic perineal, suprapubic, testicular, penile, or ejaculatory pain associated with variable dysuria and obstructive and irritative voiding symptoms |
| III A | Inflammatory | Significant number of white blood cells in expressed prostatic secretions, post-prostatic-massage urine sediment, or semen | See category III |
| III B | Noninflammatory | Insignificant number of white blood cells in expressed prostatic secretions, post-prostatic-massage urine sediment, or semen | See category III |
| IV | Asymptomatic inflammatory prostatitis | White blood cells (and/or bacteria) in expressed prostatic secretions, post-prostatic-massage urine sediment, semen, or histological specimens of prostate gland | Asymptomatic |

Table 2 Occurrence of coryneform bacteria in seminal fluid in prostatitis patients and controls

| Coryneform bacteria | Men with severe inflammation (>1 million WBC per ml of semen), n = 18 | | | Men with moderate inflammation (0.2–1 million WBC per ml of semen), n = 32 | | | Controls, n = 59 | | |
|------------------------------------|---|------------------------|------|--|------------------------|----|------------------|------------------------|-----|
| | Mean, log 10 | Median (range), log 10 | % | Mean, log 10 | Median (range), log 10 | % | Mean, log 10 | Median (range), log 10 | % |
| <i>Arthrobacter</i> sp. | 3.8 | <2 (<2–5.0) | 17* | <2 | <2 | 0 | 1.2 | <2 (<2–3.0) | 2* |
| <i>Brevibacterium</i> sp. | 3.4 | <2 (<2–4.7) | 6 | <2 | <2 | 0 | 1.9 | <2 (<2–3.7) | 2 |
| <i>Corynebacterium coyleae</i> | <2 | <2 | 0 | 1.5 | <2 (<2–3.0) | 3 | <2 | <2 | 0 |
| <i>Corynebacterium</i> group A | <2 | <2 | 0 | <2 | <2 | 0 | 1.2 | <2 (<2–3.0) | 2 |
| <i>Corynebacterium</i> group F1 | 3.7 | <2 (<2–5.0) | 6 | 2.8 | <2 (<2–4.0) | 6 | <2 | <2 | 0 |
| <i>Corynebacterium</i> group G | 3.8 | <2 (<2–5.0) | 33** | 2.5 | <2 (<2–4.0) | 3 | 0.2 | <2 (<2–2.0) | 2** |
| <i>Corynebacterium jeikeium</i> | 3.8 | <2 (<2–5.0) | 11 | 1.5 | <2 (<2–3.0) | 3 | 4.3 | <2 (<2–6.0) | 7 |
| <i>Corynebacterium mucifaciens</i> | <2 | <2 | 0 | 2.5 | <2 (<2–4.0) | 6 | 1.9 | <2 (<2–3.7) | 2 |
| <i>Corynebacterium seminale</i> | 4.9 | 2 (<2–5.7) | 61 | 4.8 | 3 | 59 | 4.5 | 2 (<2–6.0) | 58 |
| <i>Corynebacterium striatum</i> | 3.5 | <2 (<2–4.7) | 11 | 3.7 | <2 (<2–5.0) | 9 | 1.3 | <2 (<2–3.0) | 3 |
| <i>Cellulomonas/Microbacterium</i> | 0.7 | <2 (<2–2.0) | 6 | 3.8 | <2 (<2–5.0) | 9 | 1.3 | <2 (<2–3.0) | 3 |
| <i>Corynebacterium</i> sp. | 5.5 | <2 (<2–6.7) | 28 | 4.0 | <2 (<2–5.0) | 19 | 4.1 | <2 (<2–5.7) | 29 |
| <i>Dermabacter hominis</i> | <2 | <2 | 0 | <2 | <2 | 0 | 2.9 | <2 (<2–4.7) | 7 |
| <i>Gardnerella vaginalis</i> | 2.7 | <2 (<2–4.0) | 6 | <2 | <2 | 0 | 4.3 | <2 (<2–6.0) | 10 |
| <i>Turicella otitidis</i> | <2 | <2 | 0 | <2 | <2 | 0 | 0.2 | <2 (<2–2.0) | 2 |
| Catalase-negative coryneform | 4.5 | <2 (<2–4.7) | 17 | 3.5 | <2 (<2–5.0) | 22 | 4.1 | <2 (<2–5.7) | 19 |

* $p = 0.03$; ** $p = 0.0003$ (Fisher exact test).

investigated in 36 men (30 with and 6 without leukocytospermia). The samples were cultured within 1 h.

Cytologic analysis

Semen smears were made for detecting white blood cells (WBC). The smears were air-dried, Bryan-Leishman stained, and examined with the use of oil immersion microscopy (magnification: $\times 1000$) by an experienced microscopist. polymorphonuclear (PMN) leukocytes were differentiated from spermatids by the presence of segmented nuclei, bridges between lobes of nucleus, and specific granulation of the cytoplasm (Couture *et al.*, 1976). The WBC concentration in semen was calculated by using the known sperm concentration (as $10^6/\text{mL}^{-1}$) according to the following formula:

$$[\text{WBC}] = \frac{\text{number of WBCs counted}}{\text{number of sperm counted}} \times \text{semen sperm concentration.}$$

One hundred round cells were counted twice, and their mean value was registered.

Isolation and identification of coryneform bacteria

Semen and urine samples were cultured quantitatively to detect anaerobic, microaerophilic and aerobic bacteria

within 1 h from collection. Freshly prepared blood agar and chocolate agar, Wilkins-Chalgren medium (Oxoid, Unipath, Basingstoke, UK) supplemented with 5% horse blood, Wilkins-Chalgren medium supplemented with 5% horse blood and GN supplement (Oxoid), MRS agar (Oxoid) and *Gardnerella vaginalis*-selective agar (Oxoid) were used. Aerobic (blood agar) and microaerobic (chocolate agar, MRS agar, and *G. vaginalis*-selective agar in 10% CO_2 atmosphere) cultures were incubated at 37 °C for 1–3 days and anaerobic cultures (Wilkins-Chalgren media in an anaerobic glove box) for 3–5 days.

Colonies with different morphology were gram stained and examined microscopically. Primary identification of coryneform bacteria was performed by gram stain morphology and catalase test. Coryneform bacteria were found from blood agar, chocolate agar, Wilkins-Chalgren med-

ium, and *G. vaginalis*-selective agar. The semen strains were identified using the API Coryne biochemical identification system (bioMérieux, France) according to the manufacturer's instructions with the exception of *Corynebacterium seminale* strains that were identified on the basis of β -glucuronidase test on blood agar with 4-methylumbelliferyl-b-D-glucuronide (MUG) supplement (Oxoid), which

visualizes a positive reaction as a fluorescence near colonies under 254 nm ultraviolet light. In case of the urine strains only *C. seminale* was identified to the species level.

Statistical methods

Statistical analyses were performed with the use of SigmaStat (Jandel Scientific, San Rafael, CA, USA) and Excel (Microsoft Corp.) software programs. Microbial counts were compared with the Mann–Whitney rank sum test. The occurrence of microorganisms in different groups was compared with Fisher exact test.

Ethical considerations

Participation in the study was voluntary. Informed consent was obtained from the patients. The studies were approved by the Ethics Review Committee on Human Research of the University of Tartu.

Results

Coryneform bacteria were found in the semen of 38 (76%) men with inflammatory prostatitis and in 49 (83%) controls. The subjects had 0–6 (mean 1.3) different coryneforms present, 0–4 (1.4) in prostatitis patients, and 0–6 (1.2) in controls. No difference was found between NIH IIIa and NIH IV category patients (data not shown).

Altogether, 148 strains were isolated from semen samples, 120 of them were identified to species or genus level. More than half of the isolates were identified as *C. seminale*, being present in 30 prostatitis patients and 34 controls (Table 1).

Prostatitis patients harboured significantly more *Corynebacterium* group G in comparison with controls [14% (7/50) vs. 2% (1/59), $p = 0.01$]. This association was especially pronounced when the patients with severe inflammation (>1 million WBC per ml) and controls were compared [33% (6/18) vs. 2% (1/59), $p = 0.0003$]. In addition, men with severe inflammation harboured the genus *Arthrobacter* significantly more frequently than the controls [17% (3/18) vs. 2% (1/59), $p = 0.03$].

When only the coryneforms with high concentration ($\geq 10\,000$ CFU per ml) were analyzed, we found that in prostatitis patients, nine coryneforms exceeded this limit (*Arthrobacter* sp., *Brevibacterium* sp., *Cellulomonas/Microbacterium*, *Corynebacterium* group F1, *Corynebacterium* group G, *C. seminale*, *C. jeikeium*, *Corynebacterium* sp. and catalase-negative coryneforms). In controls, only the four last mentioned coryneforms exceeded this limit.

Half of the men (18/36) harboured corynebacteria in both semen and urine, 22% (8/36) of the men harboured

them in semen only and 3% (1/36) in urine only. Their total concentration was greater in semen than in urine (median 5000 vs. 100 CFU per ml) yet the difference was slightly above the significance level ($p = 0.053$). Of urine strains, only *C. seminale* was identified to species level being present in 39% of men.

Discussion

Our data showed that although coryneform bacteria are frequent inhabitants in the semen of both healthy men and those suffering from prostatitis, their species composition differs between these groups. *Corynebacterium* group G and *Arthrobacter* sp. were more commonly present in the latter. Although coryneforms are generally considered as saprophytes, they are not uniform and some species may be associated with infection in the male upper genital tract.

Coryneform bacteria have been previously found from the urogenital tract of both healthy men (Willen *et al.*, 1996) and patients with prostatitis (Riegel *et al.*, 1995; Domingue & Hellstrom, 1998; Tanner *et al.*, 1999). Based on prostatic fluid cultures, Drach (1974) observed that 12% of prostatitis cases studied were caused by coryneform organisms in pure culture or with associated bacteria. In addition to culturable coryneforms, the difficult-to-culture and non-culturable coryneforms have also been found in prostatitis patients (Domingue *et al.*, 1997; Tanner *et al.*, 1999). Domingue *et al.* (1997) found *Corynebacterium* group ANF and *C. minutissimum* from expressed prostatic secretions of prostatitis patients suggesting that cryptic coryneforms could be hidden etiologic agents in chronic prostatitis and that they are often missed or overlooked in routine clinical microbiology laboratories. Tanner *et al.* (1999) performed a thorough investigation of multiple bacteria in expressed prostatic secretions of prostatitis patients using both cultural and molecular methods and found that the most diverse and the most abundant sequences were characteristic of corynebacteria. Although only three species (*C. afermentans*, *C. xerosis*, and *Corynebacterium* group ANF) were found by culture method, the 16S rDNA sequences of 15 *Corynebacterium* spp. were identified, each patient having at least one species and one patient even nine different species. At the same time control samples had sequences indicating only three corynebacterial species, and most of the control samples lacked detectable corynebacterial sequences. The authors concluded that diverse corynebacteria thrive in abundance in the inflamed prostate, although it may not be final confirmation that they are causative agents of the disease state. Other culturable coryneform species isolated from semen or expressed prostatic secretions include *C. seminale*

(Riegel *et al.*, 1995), *C. singulare* (Riegel *et al.*, 1997), *C. freneyi* (Renaud *et al.*, 2001), *C. striatum/amycolatum*, *C. macginley*, *C. jeikeium*, *D. hominis* (Jędrzejczak *et al.*, 2005), and even *C. diphtheriae* (Machado *et al.*, 1989). *Gardnerella vaginalis*, a catalase-negative coryneform that has been strongly associated with bacterial vaginosis in women has been found in 19–26% of semen specimens (Hillier *et al.*, 1990; Virecoulon *et al.*, 2005).

Our prospective quantitative study with six media and three different environments revealed very frequent occurrence of corynebacteria in semen that corresponds to our previous studies (Kermes *et al.*, 2003; Punab *et al.*, 2003; Korrovits *et al.*, 2006) as well as to the data of Hillier *et al.* (1990) who found them in 86% of semen specimens. As regards species composition, several coryneforms found in our current study have been described in semen earlier too; however, many of them have not been described by other authors. We found *C. seminale* to be the commonest species yet no clear association with prostatitis was revealed in this study. Instead, *Arthrobacter* sp. and especially *Corynebacterium* group G were associated with inflammatory prostatitis. *Corynebacterium* group G has been isolated from a variety of clinical sources (blood, eye, skin, mitral valve, abscesses, semen, urethra) and found to be responsible for bacteremia, endocarditis, septic arthritis, catheter infections, wound infections, and eye infections (Funke *et al.*, 1997; Funke & Bernard, 2003). The genus *Arthrobacter* is widely distributed in the environment but has been also isolated from human clinical specimens such as blood, wound secretion, lymph node biopsy specimen, ocular fluid, fluid from the external ear, urine, vaginal, and cervical samples, being associated with bacteremia, endophthalmitis, Whipple disease-like syndrome, phlebitis, endocarditis, foreign body infections, and urinary tract infections (Funke & Bernard, 2003; Bernasconi *et al.*, 2004). At the same time, there is lack of knowledge regarding the virulence factors and pathomechanisms of these coryneforms. Their causal relationship with prostatitis needs to be confirmed by further studies.

Numerous coryneform species have also been found from urethra and/or urine of the patients with lower urinary tract infections (Camello *et al.*, 2003; Riemersma *et al.*, 2003), however, low counts of these species may belong to urethral microflora (Willen *et al.*, 1996; Montagnini Spaine *et al.*, 2000). In our study, contamination of semen specimens from urethra was diminished by urinating before sampling. In addition, we compared the corynebacteria of first-catch urine (that represents urethral microflora) and seminal fluid and found their counts to be much higher in semen, suggesting other sources of bacteria besides urethra.

Conclusions

Although coryneforms are generally considered as saprophytes, they are not uniform in semen. *Corynebacterium* group G and *Arthrobacter* sp. show an association with inflammatory prostatitis.

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