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Technical note

Isolation of *Arcanobacterium pyogenes* from the scrotal skin and the prepuce of healthy rams or from rams with testicular abnormalities

P.G. Gouletsou [a], G.C. Fthenakis [a,*], A. Tzora [b], P.J. Cripps [c], P. Saratsis [d]

[a] *Faculty of Veterinary Science, University of Thessaly, P.O. Box 199, 43100 Karditsa, Greece*
[b] *Department of Animal Production, TEI Epirus, P.O. Box 110, 67100 Arta, Greece*
[c] *Faculty of Veterinary Science, University of Liverpool, Neston, South Wirral CH64 7TE, UK*
[d] *School of Veterinary Medicine, Aristotle University of Thessaloniki, 54006 Thessaloniki, Greece*

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Abstract

In a field study, samples were obtained from the scrotal skin and the preputial cavity of 48 rams and examined bacteriologically. Bacteria were isolated from all skin samples and from 90% of preputial cavity samples. The most frequently isolated organisms were *Staphylococcus* spp., *Bacillus* spp., *Clostridium* spp., *Acinetobacter* spp. and *Escherichia coli*. *Arcanobacterium pyogenes* was isolated from the skin samples of 33% and the preputial cavity samples of 8% of rams. Furthermore, the clinical and pathological features of three clinical cases of testicular abnormalities associated with *A. pyogenes*, are also presented; in two animals, the organism caused long-standing orchitis and the third scrotal cellulites and testicular degeneration. These findings confirm that *A. pyogenes* can be a testicular pathogen under field conditions; the source of infection appears to be organisms present on the scrotal skin.
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1. Introduction

Orchitis is an important disorder of rams, as it affects the genitalia and results in reduced fertility. According to Bath and de Wet (2000), the organisms that may infect the genitalia of rams belong mainly to one of the following three groups: (i) *Brucella* spp., (ii) the *Haemophilus*, *Pasteurella* and *Actinobacillus* group and (iii) the *Arcanobacterium* and *Corynebacterium* group.

The majority of references in the international literature deal with the first two groups of organisms; in fact, there is little information available regarding the role of Arcanobacteria and Corynebacteria as testicular

* Corresponding author. Tel.: +30 2441 066 070; fax: +30 2441 0660 77.
E-mail address: gcf@vet.uth.gr (G.C. Fthenakis).

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pathogens. In the present article, we present (i) the results of a field investigation of the bacterial flora of the genitalia of rams and (ii) the features of three field cases of testicular disorders associated with *A. pyogenes*.

2. Materials and methods

2.1. Investigation of the bacterial flora of the genitalia of rams

The study was carried out in nine sheep flocks in Greece and 48 clinically healthy rams, aged 2–5 years. The study took place from May to September, during the reproductive season of sheep. Samples were obtained in the morning, before the animals were released into the flock of the ewes for the day. Samples were obtained from three sites in each ram; two swabs moistened into Soy-broth, were used in each site. Initially, samples were obtained with the ram in the standing position; an area of approximately 2 cm^2 was swabbed in the caudal surface of the scrotum. Subsequently, the ram was cast and restrained and two further samples were obtained; one from an area of approximately 2 cm^2 from the cranial surface of the scrotum and another after inserting the swab into the preputial cavity.

The swabs were cultured within 30–60 min of collection. One of the swabs obtained from each site, was plated onto Columbia blood agar and MacConkey agar plates; the media were subsequently incubated aerobically, anaerobically and in CO_2 environment for up to 72 h. Identification of bacteria was carried out by using conventional techniques (Barrow and Feltham, 1993; Dubreuil, 2002), as well as quick identification methods (API-System; BioMerieux SA, Marcy-l'-Etoile, France). The other swab was immersed into a sterile vial containing 2 ml of phosphate buffer saline pH 7.3 (PBS), swirled and maintained therein for 5 s. The number of bacteria in the PBS was subsequently measured by making serial dilutions according to the method of Miles and Misra (1938).

For statistical analysis, Minitab (Minitab 14, Minitab Inc., State College, PA, USA) and Stata (Stata 8, College Station, TX, USA) were used. Following basic descriptive statistical analyses, the total aerobic bacterial counts were transformed to $\log_{10}$ after first adding "one" to each reading. Results between sampling sites and rams were compared using analysis of variance and the differences between sites were assessed using Tukey's method for multiple comparisons. The number of bacterial species per site was investigated using a generalised linear model, with the response declared as a Poisson variable and a log link. The effect of site was assessed using analysis of deviance. The proportions of the following bacterial species: *A. pyogenes*, *Staphylococcus* spp., *Bacillus* spp., *Escherichia coli* and "other species" at the different sampling sites were compared using χ^2-tests, having first combined smaller categories to avoid low expected frequencies. Where appropriate the statistical significance was adjusted to take account of multiple comparisons. Statistical significance was defined as $P < 0.05$ on a two-sided null hypothesis of no difference.

2.2. Case reports: clinical cases of testicular disorders associated with A. pyogenes

Three cases of testicular abnormalities were examined in detail. From all, *A. pyogenes* was isolated in pure culture and heavy growth, when samples were bacteriologically examined.

Initially, a detailed examination of the genitalia of the rams was carried out, as described before (Fthenakis et al., 2001). Subsequently, the animals were euthanatised and a detailed post-mortem examination was carried out; by using the aseptic technique, samples were obtained for bacteriological examination. Tissue samples were also collected for histological examination; these were fixed into Bouin's solution and then cleaned in alcohol 50°; they were embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin (H&E).

3. Results

3.1. Investigation of the bacterial flora of the genitalia of rams

Bacteria were isolated from all scrotal skin samples and from 90% of preputial cavity samples. The mean number of bacterial species in samples from the caudal or cranial scrotal skin ranged from 3 to 8, with a mean value of 4.8 ± 0.2 and 4.7 ± 0.2, respectively; ($\pm$S.E. of the mean); the mean number of bacterial species in samples from the preputial cavity ranged from 0 to 3,

with a mean value of 1.2 ± 0.1. No statistical significant difference ($P > 0.05$) was found between the mean number of bacterial species in samples from the caudal or the cranial scrotal skin; however, there was a highly significant difference ($P < 0.001$) between those and the mean number of bacterial species in samples from the preputial cavity.

Total aerobic bacterial counts in swabs from the caudal scrotal skin ranged from 5.0×10^2 to 6.8×10^4 c.f.u., with a geometric mean of 5.37×10^3 c.f.u. Total aerobic bacterial counts in swabs from the cranial scrotal skin ranged from 4.3×10^2 to 6.7×10^4 c.f.u., with a geometric mean of 3.95×10^3 c.f.u. Those in swabs from the preputial cavity ranged from 0 to 2.0×10^2 c.f.u., with a geometric mean of 2.08×10^2. No statistical significant difference ($P > 0.05$) was found between counts in swabs from the caudal or the cranial scrotal skin; however, there was a highly significant difference ($P < 0.001$) between those and counts in swabs from the preputial cavity.

The most frequently isolated organisms from scrotal skin samples were *Staphylococcus* spp. (48 rams) and *Bacillus* spp. (32 rams); *A. pyogenes* was isolated from 16 rams (33%). The most frequently isolated organisms from preputial cavity samples were *Staphylococcus* spp. (16 rams) and *E. coli* (15 rams); *A. pyogenes* was isolated from samples of 4 rams (8%). Details are in Table 1. There were no significant differences in the bacterial species isolated from the caudal or cranial scrotal skin samples; however, there were significant differences ($P < 0.005$) between those isolated from either of these and those from the preputial cavity. The main contribution to this difference was an over-representation of *E. coli* in the preputial cavity and a corresponding under-representation of "other species".

3.2. Case reports: clinical cases of testicular disorders associated with A. pyogenes

3.2.1. Animals no. 1 and 3: long-standing orchitis

The first clinical case was in a 13-year-old ram, referred with a suggested reduced reproductive performance; the only clinical finding was mildly enlarged right testicle and epididymis, without a clear definition of their boundaries. The third case was in a 4-year-old ram, presented with abnormal left scrotal contents; the left testicle and epididymis were smaller and harder than the right and did not move freely into the scrotum.

Pathological findings in the first animal were as follows; in the left genitalia: decreased testicular size and enlarged mediastinum testis, increased size of epididymal tail and head; in the right genitalia: adhesions between the epididymal head and the parietal vaginal tunic, copious amount of sero-sanguineous fluid in the vaginal cavity, particularly reduced testicular size with pale parenchyma and without mediastinum testis, pale and enlarged epididymal head and particularly enlarged epididymal tail with an abscess (3.0×2.5 cm). In the third animal, lesions were limited to the left genitalia; testicular size was reduced, the parenchyma was hard, pale and surrounded by a hard capsule, with no mediastinum evident. The epididymis was enlarged and pale, with adhesions between the visceral and the parietal vaginal tunic. In bacteriological examination, *A. pyogenes* was isolated in pure culture and heavy growth.

In the left testicular parenchyma of the first animal, there was marked fibrosis with loss of architecture, collapsed seminiferous tubules with cell sloughing and multinucleated giant cell formation and presence of few spermatozoa; there was fibrosis with loss of architecture in the left epididymis; the duct contained only proteinaceous material with no epithelium present, although in one section a large number of spermatozoa was evident. In the right testicular parenchyma, there was very marked fibrosis with total loss of architecture, almost devoid of seminiferous tubules, except of few collapsed tubules; no Leydig cells were apparent; there was marked fibrosis with necrosis of the epithelium of the right epididymal duct and no spermatozoa were recognized.

There was a thick layer of fibrous tissue surrounding the left testicular parenchyma of the third animal; seminiferous tubules were mineralized or markedly degenerated with almost complete cell sloughing and formation of multinucleated giant cells; no Leydig cells were observed. There was marked fibrosis in the left epididymis, with the epithelium of the duct being shorter, wrinkled and vacuolated and no spermatozoa visible.

3.2.2. Animal no 2: scrotal cellulitis and degeneration of testicular parenchyma

The second clinical case was in a 4-month-old ram, presented with an enlargement in the penile area and inability to urinate. On clinical examination we found the whole genitalia to be particularly enlarged, whilst

Table 1
Frequency of isolation of each bacterial species from samples obtained from scrotal skin or the preputial cavity of rams

Bacterial genera or species	Number and proportion of isolates from		
	Caudal scrotal skin	Cranial scrotal skin	Preputial cavity
Coagulase-negative *Staphylococcus* spp.	52 (22.6%)	50 (22.3%)	16 (27.1%)
S. epidermidis	11	8	5
S. simulans	10	12	4
S. caprae	7	8	1
S. sciuri	7	6	0
S. chromogenes	6	4	0
S. xylosus	6	6	1
S. auricularis	0	1	0
Staphylococcus spp.	5	5	3
Bacillus spp.	31 (13.5%)	32 (14.3%)	7 (11.9%)
B. licheniformis	17	23	3
B. cereus	3	2	0
Bacillus spp.	11	7	4
Clostridium spp.	15 (6.5%)	14 (6.3%)	2 (3.4%)
C. perfringens	7	9	0
Clostridium spp.	8	5	2
Acinetobacter spp.	15 (6.5%)	11 (4.9%)	0 (0.0%)
A. calcoaceticus	7	7	
A. baumanii	3	0	
Acinetobacter spp.	5	4	
Escherichia coli	15 (5.7%)	16 (7.1%)	15 (25.4%)
A. pyogenes	13 (5.7%)	11 (5.4%)	4 (6.8%)
Erwinia spp.	13 (5.2%)	14 (6.3%)	0 (0.0%)
Micrococcus spp.	12 (5.7%)	14 (6.3%)	3 (5.1%)
M. roseus	7	9	3
Micrococcus spp.	6	5	0
Peptoniphilus indolicus	10 (4.3%)	11 (4.9%)	0 (0.0%)
Corynebacterium spp.	7 (3.0%)	6 (2.7%)	4 (6.8%)
C. pseudotuberculosis	7	7	0
C. glucuronolyticum	0	0	4
Mannheimia haemolytica	7 (3.0%)	8 (3.6%)	0 (0.0%)
Fusobacterium necrophorum subsp. *necrophorum*	6 (2.6%)	6 (2.7%)	0 (0.0%)
Staphylococcus aureus	6 (2.6%)	9 (4.0%)	1 (1.6%)
Streptococcus spp.	6 (2.6%)	7 (3.1%)	3 (5.1%)
S. uberis	2	3	1
S. ovis	1	0	0
Streptococcus spp.	3	4	2
Kokuria rosea	5 (2.3%)	4 (1.80%)	0 (0.0%)
Pasteurella multocida	5 (2.3%)	5 (2.2%)	2 (3.4%)
Actinobacillus lignieresii	4 (1.7%)	1 (0.4%)	2 (3.4%)
Burkholderia cepacea	4 (1.7%)	3 (1.3%)	0 (0.0%)
Proteus spp.	4 (1.7%)	2 (0.9%)	0 (0.0%)
Total	230	224	59

the scrotal contents could not be palpated; furthermore, two cysts on the scrotal skin, extreme swelling in the area laterally to the prepuce and penis and inability to extrude the penis were evident. Pathological findings included presence of sero-sanguineous fluid within the scrotum and between the various scrotal tunics, darkly tanned mediastinum testis, urethral rupture and peri-urethral haematoma in the area immediately adjacent to the genitalia. *A. pyogenes* was isolated from the fluid.

There was mild degeneration of the seminiferous epithelium with vacuolation in the parenchyma of both testicles; spermatogenesis was not complete and no spermatozoa were evident; some seminiferous tubules had erythrocytes in their lumen and *rete testis* was filled by erythrocytes. The epididymides appeared normal. There was oedema and neutrophil aggregation within the scrotal skin.

4. Discussion

A. pyogenes has been found to cause various infections in sheep; it has been isolated from cases of pneumonia, mastitis, metritis, abortion and abscesses in various parts of the body (Euzeby, 1997). In a previous paper (Gouletsou et al., 2004), we described in detail the features of experimentally induced orchitis caused by *A. pyogenes*. We proposed that the organism might invade the testicles consequently to a microbial or physical trauma to the underlying tissues. The frequent isolation of *A. pyogenes* from samples obtained from the scrotal skin of rams, lends support to our theory. However, the transition of the organism from commensal to pathogenic state is not clear (Billington et al., 2001).

P. indolicus, which was also isolated from scrotal skin samples, is part of the skin flora of cattle. The synergistic action of this organism with *A. pyogenes* in causing dry-cow mastitis has been documented (Seinhorst et al., 1991; Madsen et al., 1992). Both organisms have been isolated from the flies *Hydrotaea irritans* and *Stomoxys calcitrans*, which abound throughout Europe during the summer, and which are considered to transmit the bacteria between animals (Chirico et al., 1997). Madsen and others (1992) have found that during the summer there was an increase in the infection rate with these two organisms. One may thus suggest, that the organisms may also be transmitted to sheep, especially as the reproductive period is usually during the summer, when sheep are at pasture; consequently, chances of infection of the animals are greater.

Furthermore, injuries to the scrotum, which are common in rams during the summer (e.g. as a result of kicks from other rams), could also have contributed. Seinhorst et al. (1991) found that teat injury, which compromises the defence mechanisms of the teat (Geishauser and Querengasser, 2002), should be experimentally inflicted, in order to achieve colonization of the mammary gland by *A. pyogenes* and mastitis. Similarities between the above models and the situation in ovine orchitis are possible.

No differences were evident in the number of bacterial species and in the total number of aerobic bacteria isolated from the caudal or the cranial surface of the scrotum. The caudal surface is exposed and can be easily contaminated (e.g. from faeces), whilst the cranial surface may be contaminated from bacteria transferred from the perineal area of the ewe during mating. As expected, coagulase-negative staphylococci were the most frequently isolated bacteria; these were also the most frequently isolated bacteria from samples obtained from udder skin of ewes (Fthenakis, 1988).

The isolation of some of the other organisms is noteworthy. *A. baumanii* has thusfar been isolated only from nosocomial infections of horses, cats, dogs (Boerlin et al., 2001), but never from sheep. *S. ovis* had previously been isolated from pathological conditions of sheep, always in a mixed culture with *A. pyogenes* or *F. necrophorum* subsp. *necrophorum* (Collins et al., 2001); this could suggest that the organism may also cause a pathological condition to the genitalia. *C. glucuronolyticum* is considered to be a part of the normal flora of men and boars (Devriese et al., 2000), but had never been isolated from rams. *M. haemolytica* is a member of the bacterial flora of the upper respiratory tracts of sheep; its isolation from scrotal skin samples may be a consequence of the breeding habits of sheep, as ewes nose and "nudge" the testicles of rams (Odagiri et al., 1995) before mating; in the process bacteria might have been transferred from the nasopharynx of the ewes to the scrotum of rams, as it has already been suggested for lambs transferring the organism to the teats of their dams (Mavrogianni et al., 2005).

Histological examination of tissue samples from animals no. 1 and 3 revealed lesions typical of long-standing orchitis; these were likely the consequence

of the pathogenic action of *A. pyogenes*, as some of these (e.g. degeneration of seminiferous tubules, interluminary presence of leucocytes, destruction of Leydig cells) had already been described during experimentally induced orchitis (Gouletsou et al., 2004). In tissue samples from animal no. 2, *A. pyogenes*, likely from the preputial cavity or the scrotal skin, invaded the tissues and grew into the urine consequently to urethral rupture. The degeneration of the seminiferous epithelium observed might have been the result of the direct action of the organism or else, it was the result of increased temperature and fluid accumulation around the testicles. These findings fulfill "Koch's postulates" and thus confirm that *A. pyogenes* is pathogenic for the testicles of rams.

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