

A STUDY FOR THE DIFFERENTIATION OF *ACTINOBACILLUS SEMINIS*, *A. ACTINOMYCETEM-COMITANS*, *HISTOPHILUS OVIS* AND *PASTEURELLA HAEMOLYTICA*

MARTHA L. SWANEPOEL, Veterinary Research Institute, Onderstepoort 0110

ABSTRACT

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By using well-defined techniques under optimum conditions it is possible adequately to define the biochemical characteristics of typical *A. seminis* strains. *A. seminis* can be distinguished from *Histophilus ovis* on the latter's distinctive colony morphology, but it cannot be distinguished from *Actinobacillus actinomycetem-comitans*. These organisms, however, can be differentiated from *Pasteurella haemolytica* on serological grounds and the latter's greater pathogenicity for mice. It is appreciated, however, that intermediate forms occur which cannot as yet be satisfactorily allocated to any of the above-mentioned genera.

INTRODUCTION

Actinobacillus seminis was first isolated in Australia (Baynes & Simmons, 1960). Many publications have dealt with the isolation of similar gram-negative pleomorphic bacteria (Dodd & Hartley, 1955; Ekdahl, Money & Martin, 1968; Pulsford, Eastick, Clapp & Roberts, 1967; Watt, 1966; Watt, Bamford & Nairn, 1970), and in South Africa 6 strains of *A. seminis* were isolated and described by Van Tonder (1979). It became clear from the literature that authors could not say with certainty that an isolate was *A. seminis* or not. Despite the various descriptions, the characteristics of *A. seminis* have not yet been adequately defined. This group of organisms does not grow well on the conventional media used for biochemical tests, and as a result of the weak biochemical reactions appreciable difficulty still exists in the differentiation of *A. seminis*-like organisms from *Pasteurella* spp.

Baynes & Simmons (1960) described certain characteristics for *A. seminis*. In subsequent reports, based on single strains, some of these characteristics were confirmed (Van Tonder & Bolton, 1968), but certain differences were also found (Livingstone & Hardy, 1964; Worthington & Bosman, 1968; Van Tonder, 1979).

The object of this investigation was to study the growth characteristics, morphology, pathogenicity, certain serological properties and biochemical features of a number of recognized *A. seminis* strains. By establishing optimum conditions for conducting biochemical tests an attempt was made to establish firm criteria for the definite identification of *A. seminis* and to enable a clear distinction to be made between *A. seminis*, *Histophilus ovis*, *P. haemolytica* and other gram-negative pleomorphic bacteria commonly found in the genital tract of rams.

MATERIALS AND METHODS

Cultures⁽¹⁾

An *A. seminis* strain, K3844C, similar to the one described by Baynes & Simmons (1960), was used. The 4 strains, T981V, U207, 70.64 and 6201, isolated in the Middelburg District, Cape Province, conformed to Van Tonder's (1979) description of *A. seminis*. In the same investigation strains 1032A and V350, which resembled *H. ovis*, were isolated by Van Tonder (1979). Two *P.*

haemolytica strains were used, a Type 1, which fermented arabinose, but not trehalose, and a Type 3 which was able to utilize the trehalose, but not arabinose. A strain of *A. actinomycetem-comitans* was also included in the study.

Growth characteristics

Colony morphology was studied by observing growth on bovine blood tryptose agar for 24-72 h. Gram-stained smears were used to study microscopic morphology. Haemolysis was studied on sheep and bovine blood tryptose agar incubated for 24-48 h.

MacConkey's agar⁽²⁾ was used to study the ability of the different strains to grow on this medium. Agar plates were incubated for 72 h aerobically and in an atmosphere with 10% CO₂.

Biochemical tests

Carbohydrate fermentation. Peptone water was used as the basal medium and was prepared with and without Na₂HPO₄. The medium without Na₂HPO₄ was prepared according to the method described by Cruickshank, Dui-guid, Marmoin & Swain (1975). For the basal medium with Na₂HPO₄, 2 g of Na₂HPO₄.12H₂O was added to 1 ℓ of peptone water. The pH of the basal media was adjusted to 7.3-7.4.

Andrade's indicator was added to the peptone water to give a final concentration of 1% and bromocresol purple was added to give a final concentration of 0.0005%. The influence of serum on fermentation was tested by adding sterile sheep serum to the basal medium at a 10% concentration.

The fermentation of glucose was studied under different conditions to determine the optimum conditions for the detection of acid production. Tests were read after 2, 5 and 7 days incubation at 37 °C. If no colour change occurred during this period, the test was read as negative. The fermentation of 3 other carbohydrates, maltose, arabinose and trehalose, was carried out according to the optimum conditions for glucose fermentation. The carbohydrates were added to a final concentration of 1%.

Nitrate reduction

Nitrate reduction tests were carried out in 3 different media; a solid medium (Society of American Bacteriologists, 1957), a semi-solid medium (Finegold, Martin & Scott, 1962) and a liquid medium (Edwards & Ewing, 1968). Results were recorded after 2, 5 and 7 days.

Arginine dihydrolase test and ornithine decarboxylase test

The arginine dihydrolase medium was prepared as an arginine broth (Harrigan & McCance, 1966) and the

⁽¹⁾ *A. seminis*, strain K3844C, was obtained from M. D. Connole, Animal Research Institute, Queensland, Australia, and *A. actinomycetem-comitans* from W. J. de Long, College of Agriculture, University of Idaho, U.S.A. *P. haemolytica* types 1 and 3 was obtained from E. L. Biberstein, University of California, Davis, California. Strains T981V, U207, 70.64, 6201, 1032A and V350 were obtained from E. M. van Tonder, Regional Veterinary Laboratory, Middelburg, C.P.

ornithine decarboxylase medium was prepared as described by Finegold *et al.* (1962). Results were recorded after 2, 5 and 7 days.

Antibiotic sensitivity

Tests for antibiotic sensitivity were carried out, using Mastring-S antibiotic discs⁽³⁾. Blood tryptose agar plates were heavily inoculated from broth cultures. The agar plates were allowed to dry and the discs were put onto the agar. Zones of inhibition were arbitrarily scored after 24 and 48 h as zero to 4, with zero as no inhibition and 4 a wide zone. The antibiotics used were penicillin G 1 unit, chloramphenicol, streptomycin, tetracycline and neomycin 1 µg, kanamycin 30 µg and erythromycin 5 µg.

Serology

Preparation of antisera. Antisera were prepared by a modified version of the method of N. J. L. Gilmour, Moredun (personal communication to Dr C. M. Cameron, VRI, Onderstepoort, 1978). Serotypes were harvested from the surface of 18 h sheep blood agar cultures by washing off the growth with 0.3 % formol saline. Suspensions in pure saline were standardized to Brown's opacity tube 2 [approximately 10⁸ organisms(orgs)/ml] and incubated for 2 h at 37 °C. Rabbits were given 6 injections at 3-day intervals. The first injection was 0.3 ml subcutaneously, followed by 0.5 ml intravenously (i/v). Subsequent injections of 1.0 ml, 1.0 ml, 2.0 ml with a final 1.0 ml i/v were used. Rabbits were bled from the marginal ear vein 10–14 days after the last injection. If titres of 1:160 or higher were obtained, the rabbits were exsanguinated.

Haemagglutination tests. These tests were performed as described by Carter (1955), except that guinea-pig erythrocytes were used instead of human O-erythrocytes.

Precipitin tests. These tests were performed according to the method described by Cameron (1966). The method for phenol extraction of capsular material was based on the procedure described by Sutherland & Wilkenson (1971). An adaptation of this method by Cameron, Pienaar & Vermeulen (1980) was used in this study as only small quantities of extracts were required for precipitin tests.

Tube agglutination tests. The method used for tube agglutination was that described by Cameron, Botha & Smit (1976).

Pathogenicity tests

The pathogenicity to mice of the various strains was tested by injecting mice, approximately 6 weeks old, intraperitoneally with 3 different concentrations of the bacteria. The organisms were suspended in tryptone water to give a reading of 24 on the linear scale of an Eel Unigalvo Nephelometer⁽⁴⁾. A reading of 24 contained about 3.5 × 10⁸ orgs/ml. These suspensions were diluted 1/5 and 1/25. One part of each suspension was mixed with 4 parts mucin. Ten mice were injected with 0.5 ml of each suspension.

Control mice were injected with tryptone water and mucin only. The number of dead mice was recorded after 3 days.

The mucin was prepared as follows: 50 g of mucin (BDH)⁽⁵⁾ was blended with 1 l of distilled water. The solution was steamed at atmospheric pressure for 30 min and filtered through 3 layers of gauze. The solution was steamed again for 30 min at atmospheric pressure on 3

consecutive days and stored at 4 °C until required. The required quantity was boiled for 5 min and the pH adjusted to 7.2–7.4. Sterile glucose was added to the mucin to a final concentration of 1 %. The solution was boiled again for 5 min and cooled before use.

RESULTS

Growth characteristics

Colony morphology. The various strains were able to grow aerobically, but optimum growth occurred only in a carboxyphylic atmosphere. Differences between the colonies of the various strains were most apparent on blood tryptose agar incubated in an atmosphere with 10 % CO₂. Little difference could be seen between colonies of the various strains after 24–48 h incubation. More apparent differences like colony form, edge and profile could be observed after 72 h.

Colonies of *A. seminis* on blood tryptose agar had a grey-white colour and in young cultures were round, low convex with an entire edge. After 72 h incubation the colonies had an uneven shape with an undulating edge and umbonate profile. The colonies had a butyrous consistency. The colonies of *A. actinomycetem-comitans* were similar to those of *A. seminis*, but had a smoother consistency. The colony morphology of strains T981V, U207, 70.64 and 6201 were in all respects similar to the colonies of *A. seminis* (K3844C).

Colonies of the 2 *P. haemolytica* strains were larger than those of the actinobacilli. In young culture the colonies were flat and round with entire edges. Colonies of older cultures were convex and round with undulating edges. The consistency varied from smooth to mucoid or butyrous.

Colonies of strains 1032A and V350 were found to be markedly different from the colonies of the other organisms studied. They were much smaller, had a yellow colour, were flat and round, with entire edges, and had a smooth to butyrous consistency.

Microscopic morphology. The organisms studied were mostly of coccobacillary form with a length of 1 µm. Shorter and longer bacilli and occasional cocci and filaments did occur. Individual organisms of strains 1032A and V350 were smaller than those of the actinobacilli.

Haemolysis. *A. seminis*, *A. actinomycetem-comitans* and the strains T981V, U207, 70.64 and 6201 were mostly non-haemolytic, but showed some alpha-haemolysis. Strains 1032A and V350 were alpha-haemolytic. *P. haemolytica* type 3 was beta-haemolytic, while type 1 showed occasional alpha-haemolysis or none. The use of sheep or bovine blood for the tryptose agar plates and aerobic or carboxyphylic incubation had no influence on the pattern of haemolysis.

Growth on MacConkey's agar. Only the 2 strains of *P. haemolytica* were sometimes able to grow on the medium. Growth appeared after 24 h as small, pink, flat to convex colonies with entire edges.

Fermentation

The best medium to use for fermentation tests by this group of organisms was peptone water without added Na₂HPO₄ or serum, with Andrade's as pH indicator and incubated in a carboxyphylic atmosphere. With added serum or Na₂HPO₄ or the use of Bromocresol purple as indicator, acid production was observed at a much later time, or not at all. Glucose and maltose were readily fermented by all the strains and arabinose and trehalose to a lesser extent.

⁽³⁾ Mast Laboratories Ltd, Liverpool, England

⁽⁴⁾ Evan Electroelenium Ltd, Halstead, Essex, England

⁽⁵⁾ Hickmann & Kleber, P.O. Box 4397, Johannesburg, 2000

Nitrate reduction tests

A. seminis, *A. actinomycetem-comitans* and the strains U207, 1032A, V350, 70.64 and 6201 were unable to reduce nitrates in the liquid medium, while the 2 *P. haemolytica* strains gave positive results. Strain T981V showed occasional positive reactions (Table 1). The solid medium gave variable results. In the semi-solid medium, all strains, except V350, reduced nitrates readily. Occasional negative reactions sometimes occurred, especially in media incubated aerobically.

Ornithine decarboxylase and arginine dihydrolase tests

None of the organisms studied were positive for arginine dihydrolase. The 2 strains of *P. haemolytica* and V350 were negative for ornithine decarboxylase, while 1032A did show positive reactions. *A. seminis*, *A. actinomycetem-comitans*, T981V, U207, 70.64 and 6201, were able to decarboxylate ornithine, but negative reactions sometimes occurred (Table 2).

Antibiotic sensitivity

The sensitivity patterns of the various strains were

such that they did not contribute to the differentiation of the organisms studied.

Serology

Tube agglutination. The titres of the different antisera presented in Table 3 were inconclusive. Many cross-reactions occurred between the antisera of the various strains.

Haemagglutination and precipitin tests. The 2 *P. haemolytica* strains appeared to be markedly different from the other organisms studied. They were the only 2 strains to show precipitin bands and they gave markedly higher titres in the haemagglutination tests (Table 4).

Pathogenicity

The 2 *P. haemolytica* strains were pathogenic to most of the mice, despite the dilution used (Table 5). The other strains were pathogenic to a smaller number of mice, only when undiluted (8×10^7 organisms/mouse) suspensions were used.

The criteria that could be used to differentiate the various strains as well as the reactions of the different groups are summarized in Table 6.

TABLE 1 The reduction of nitrate by the strains studied in 3 different media

| Medium | Organisms | | | | | | | | | |
|----------------------------------|-------------------|----------------------------------|-------|----------------|----------------|-------|-------|------|------------------------------|------------------------------|
| | <i>A. seminis</i> | <i>A. actinomycetem-comitans</i> | T981V | U207 | 70.64 | 6201 | 1032A | V350 | <i>P. haemolytica</i> type 1 | <i>P. haemolytica</i> type 3 |
| Solid medium 10% CO ₂ | + | + | + | - | - | + | - | - | + | 2-5+ |
| | ± | - | + | - | + | ± | ± | - | + | + |
| Semi-solid 10% CO ₂ | 2-14+ | 2-21+ | 2-5+ | 2-7+ | 2-14± | 2-21+ | ± | - | + | + |
| | ± | ± | + | + ⁵ | + ⁵ | + | + | - | + | + |
| Liquid 10% CO ₂ | - | - | ± | - | - | - | - | - | + | + |

2-21

Symbols: - Negative; + positive after 48 h; + positive after 2-21 days; +⁵ positive after 5 days
± More frequently negative than positive

TABLE 2 The ornithine decarboxylase and arginine dihydrolase tests incubated with 10% CO₂

| | | Organisms | | | | | | | | | |
|-------------------------|--------|-------------------|----------------------------------|-------|------|-------|------|-------|------|------------------------------|------------------------------|
| | | <i>A. seminis</i> | <i>A. actinomycetem-comitans</i> | T981V | U207 | 70.64 | 6201 | 1032A | V350 | <i>P. haemolytica</i> type 1 | <i>P. haemolytica</i> type 3 |
| Ornithine decarboxylase | 2 days | -/(+) | -/+ | (+) | +/- | -/+ | - | - | - | - | - |
| | 5 days | (+/-) | +(+) | + | (+) | -/+ | - | -/+ | - | - | - |
| | 7 days | +/- | + | + | + | -/+ | +/- | -/+ | - | - | - |
| Arginine dihydrolase | 2 days | - | - | - | - | - | - | - | - | - | - |
| | 5 days | - | - | - | - | - | - | - | - | - | - |
| | 7 days | - | - | - | - | - | - | - | - | - | - |

Symbols: - Negative; + positive; (+) weak positive; -/+ variable

TABLE 3 Reciprocal titres of homologous and heterologous antisera obtained with the tube agglutination tests

| Antigens | Antisera | | | | | | | | | |
|----------------------------------|-------------------|----------------------------------|-------|------|-------|------|-------|------|------------------------------|------------------------------|
| | <i>A. seminis</i> | <i>A. actinomycetem-comitans</i> | T981V | U207 | 70.64 | 6201 | 1032A | V350 | <i>P. haemolytica</i> type 1 | <i>P. haemolytica</i> type 3 |
| <i>A. seminis</i> | 320 | 160 | 10 | 160 | — | 10 | 160 | 160 | — | 5 |
| <i>A. actinomycetem-comitans</i> | 10 | 160 | — | 80 | 10 | 20 | 10 | 5 | — | 10 |
| T981V | 320 | 320 | 20 | 80 | 20 | 20 | 20 | 40 | 5 | 5 |
| U207 | 5 | 5 | — | 160 | 80 | — | — | 5 | — | — |
| 70.64 | 160 | 80 | 160 | 160 | 80 | 160 | 40 | 160 | 10 | 20 |
| 6201 | 40 | 20 | — | 10 | — | 160 | 20 | 80 | 5 | — |
| 1032A | 10 | 640 | 10 | 160 | — | 80 | 640 | 80 | — | — |
| V350 | 5 | 80 | 20 | 10 | 5 | — | 320 | — | 20 | 80 |
| <i>P. haemolytica</i> type 1 | — | 20 | 10 | 5 | 10 | — | — | — | — | — |
| <i>P. haemolytica</i> type 3 | 5 | 5 | 10 | 5 | 5 | — | 80 | — | — | 160 |

TABLE 4 Reciprocal titres of homologous and heterologous antisera in the haemagglutination (HA) and precipitin tests

| Antigens | Antisera | | | | | | | | | | | | | | | | | | | |
|---------------------------------------|-------------------|-----|----------------------------------|-----|-------|-----|------|-----|-------|-----|------|-----|-------|-----|------|-----|------------------------------|-----|------------------------------|-----|
| | <i>A. seminis</i> | | <i>A. actinomycetem-comitans</i> | | T981V | | U207 | | 70.64 | | 6201 | | 1032A | | V350 | | <i>P. haemolytica</i> type 1 | | <i>P. haemolytica</i> type 3 | |
| | HA | ppt | HA | ppt | HA | ppt | HA | ppt | HA | ppt | HA | ppt | HA | ppt | HA | ppt | HA | ppt | HA | ppt |
| <i>A. seminis</i> | 2 | — | 2 | — | — | — | 2 | — | 2 | — | 2 | — | 2 | — | — | — | — | — | — | — |
| <i>A. actinomycetem-comitans</i> | 2 | — | 2 | — | — | — | 2 | — | 2 | — | 2 | — | 2 | — | — | — | — | — | — | — |
| T981V | 16 | 8 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| U207 | 8 | 8 | 2 | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 70.64 | 2 | 16 | 4 | 4 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 6201 | 2 | 4 | 2 | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 1032A | 2 | 64 | 2 | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| V350 | 2 | 8 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| <i>Pasteurella haemolytica</i> type 1 | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| <i>P. haemolytica</i> type 3 | 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |

ppt = Precipitin tests

TABLE 5 Pathogenicity of various organisms in gastric mucin for mice

| Dosage per mouse | Controls | Organisms | | | | | | | | | |
|-----------------------|----------|---|----------------------------------|-------|------|-------|------|-------|------|---------------------------------------|------------------------------|
| | | <i>A. seminis</i> | <i>A. actinomycetem-comitans</i> | T981V | U207 | 70.64 | 6201 | 1032A | V350 | <i>Pasteurella haemolytica</i> type 1 | <i>P. haemolytica</i> type 3 |
| | | Number of mice dead/10,72 h after injection | | | | | | | | | |
| $\pm 8 \times 10^7$ | | 1 | 5 | 2 | 2 | 3 | 3 | 3 | 1 | 9 | 9 |
| $\pm 1,5 \times 10^7$ | | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 9 | 7 |
| $\pm 3 \times 10^6$ | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 9 | 9 |

TABLE 6 Summary of tests that can be used to differentiate the organisms studied

| Tests | Organisms | | | |
|---------------------------------------|-------------------|----------------------------------|----------------|-----------------------|
| | <i>A. seminis</i> | <i>A. actinomycetem-comitans</i> | <i>H. ovis</i> | <i>P. haemolytica</i> |
| Nitrate reduction (semi-solid medium) | + | + | +/- | + |
| Ornithine decarboxylase test | + | + | +/- | - |
| Haemagglutination test | - | - | - | + |
| Precipitin test | - | - | - | + |
| Pathogenicity to mice | - | - | - | + |
| Haemolysis | - | - | + | + |
| Growth on MacConkey's agar | - | - | - | + |

Symbols: - Negative reaction; + positive reaction; +/- variable reaction

DISCUSSION

The colony morphology of young cultures could not be used to differentiate the various organisms, but in older cultures differences became apparent that could be used to differentiate *A. seminis* and *P. haemolytica*. The colonies of strains 1032A and V350 could easily be differentiated from the other strains studied.

The actinobacilli showed occasional alpha-haemolysis, while Van Tonder (1979), using the same strains, was unable to show any haemolysis. A set pattern of haemolysis could only be observed for strains 1032A, V350 and *P. haemolytica* strains 1 and 3. Non-haemolytic strains of *P. haemolytica* sometimes occur, and thus haemolysis could not be used to differentiate *A. seminis* and *P. haemolytica*.

The 2 strains of *P. haemolytica* were the only 2 organisms that were sometimes able to grow on MacConkey's agar. Baynes & Simmons (1960) and Van Tonder (1979) also showed that *A. seminis* did not grow on this medium. Organisms that are able to grow on MacConkey's agar could be classified as *P. haemolytica* and not as *A. seminis*. However, some strains of *P. haemolytica* are unable to grow on MacConkey's agar and therefore this medium could not be used to differentiate *A. seminis* and *P. haemolytica*, or *H. ovis* in all instances.

The actinobacilli and strains 1032A and V350 were able to ferment the 4 carbohydrates which were studied in a relatively short time (4 days), when a suitable medium and optimum conditions were obtained. This is contrary to the findings of Bynes & Simmons (1960), Livingstone & Hardy (1964) and Van Tonder (1979). It can therefore be assumed that the group of organisms studied may be able to ferment other carbohydrates as well, using the

described media and conditions. *A. actinomycetem-comitans* was unable to ferment trehalose. The fermentation patterns of the 4 carbohydrates studied could not be used to differentiate *A. seminis*, *P. haemolytica* and *H. ovis*.

The best medium for nitrate reduction tests is the semi-solid medium, since it gave the most reliable results of the 3 media tested. The actinobacilli were able to reduce nitrates, but occasional negative reactions occurred. Van Tonder (1979) and Worthington & Bosman (1968) stated that nitrate reduction by *A. seminis* strains was a variable characteristic and depended on the strains. It became clear from this study that the composition of the nitrate reduction medium played a major role when this ability was studied. The inconclusive results obtained using the solid medium illustrate the variable reactions that can occur when an unsuitable medium is used.

The tube agglutination test did not show any serological differences between the various strains. This test cannot therefore be applied to differentiate the members of this group of organisms. Precipitin lines were produced only by the 2 *P. haemolytica* strains, and the titres of their antisera in the haemagglutination tests were also markedly higher than those of the other strains. These 2 serological tests could be used to differentiate the various strains, but untypable strains of *P. haemolytica* are also known to occur (Aarsleff, Biberstein, Shreeve & Thompson, 1970).

The actinobacilli and strains 1032A and V350 were only pathogenic to mice when approximately 10^8 organisms were injected, whereas the *P. haemolytica* strains were highly pathogenic at a concentration of 3×10^6 organisms.

It can be assumed that field strains may exist which will not fit precisely the description of *A. seminis*, *P. haemolytica* or *H. ovis*. An extensive comparative study of gram-negative, pleomorphic bacteria occurring in the genital tract of rams, using the techniques and criteria described in this paper, is thus indicated.

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