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DNA FINGERPRINTING OF *HAEMOPHILUS SOMNUS, HISTOPHILUS OVIS* AND *ACTINOBACILLUS SEMINIS*

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A thesis presented for the degree of Doctor of Philosophy
in the Faculty of Veterinary Medicine,
University of Glasgow.

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November, 1997.

DEDICATION

This three year study is dedicated to the wishes of my deceased father Simon Appuhamy and my deceased eldest brother Berty Ancel.

ACKNOWLEDGEMENTS

I am indebted to my supervisors Dr. Roger Parton and Dr. John G. Coote for their excellent supervision, enthusiastic encouragement and invaluable cooporation and patience during the course of this study. I also owe a debt of gratitude to them for their support in identifying funding for the third year. My supervisors for the Faculty of Veterinary Medicine, Dr. H. A. Gibbs (early part) and Dr. D. J. Taylor (latter part) are greatly acknowledged for their excellent guidence and help in this study.

It was indeed a pleasure to have collaborated with Dr. J. Chris Low, SACVS, Edinburgh. The materials and ideas which he provided added to the success of this study.

My special thanks should go to Dr. Rob Aitken for his readiness to support me in any matter i.e. academic, computer or personal.

I am greatly thankful to Emeritus Professor A. C. Wardlaw (former Head of Dept. of Microbiology) for accepting me as a student and Professor R. S. Phillips, Head of Division of Infection and Immunity, for his great support to find funds for the third year.

I wish to express my gratitude to:

Drs. Neil and Ajantha Horadagoda, University of Peradeniya, Sri Lanka, for their encouragement of throughout my academic career,

Dr. Harry Birkbeck, Infection and Immunity, for constructive suggestions,

Dr. Veer Math, Molecular Biology Support Unit, IBLS, for the provision of equipment, primers for PCR and kindness,

Dr. Jonathan Sheps, Molecular Genetics, IBLS for the support in DNA sequence analysis,

Mr. M. J. A. Mylne, Edinburgh Genetics for the provision of ram semen and cooperation.

I am grateful to Dr. M. C. L. de Alwis, former Director, Veterinary Research Institute, Sri Lanka, for his concern about our postgraduate studies and nominating me for a two years overseas research fellowship to the Sri Lanka Agricultural Research Project and to the Government of Sri Lanka for granting me leave of absence. I am also grateful to Professor J. R. Coggins, Director of Graduate School, IBLS, for providing me a full scholarship for the third year of my study.

I am also thankful for postdocs, postgraduates and staff both technical and administrative for their willingness for help.

I sincerely thank my mother, Josaphinhamy, my brothers and my sister whose good wishes enabled me to complete this study. I gratefully acknowledge the support of my wife Manjula who had to bear a heavy burden of family matters and son, Praveen for isolation of countless hours.

PRESENTATIONS / PUBLICATIONS

1. **Appuhamy, S., Parton, R., Coote, J. G. and Gibbs, H. A. (1996).** DNA fingerprinting of *Haemophilus somnus* by polymerase chain reaction. Association of Veterinary Teachers and Research Workers (Scotland), Veterinary School, Glasgow University.

2. **Appuhamy, S., R. Parton, J. G. Coote and H. A. Gibbs. (1997).** Genomic fingerprinting of *Haemophilus somnus* by a combination of PCR methods. Journal of Clinical Microbiology **35:**288-291.

3. **Appuhamy, S., Parton, R., Coote, J. G., D. J. Taylor and Low, J. C. (1997).** Identification, fingerprinting and detection of *Actinobacillus seminis* by PCR methods. 51st Scientific Meeting of Association of Veterinary Teachers and Research Workers, Scarborough, UK.

4. **Appuhamy, S., Coote, J. G., Low, J. C. and Parton, R.** PCR methods for rapid identification and characterisation of *Actinobacillus seminis* strains. Journal of Clinical Microbiology (in press).

5. **Appuhamy, S., Low, J. C., Parton, R. and Coote, J. G. (1997).** The Genbank accession number AF013275 for the nucleotide sequence of *Actinobacillus seminis* 16S and 23S ribosomal RNA genes, partial sequence, Glu-tRNA gene, complete sequence. National Center for Biotechnology Information, National Library of Medicine, Bethesda, USA.

6. **Appuhamy, S., Low, J. C., Parton, R. and Coote, J. G. (1997).** The Genbank accession number AF013276 for the nucleotide sequence of *Actinobacillus seminis* 16S and 23S ribosomal RNA genes, partial sequence, Ile-tRNA and Ala-tRNA genes, complete sequence. National Center for Biotechnology Information, National Library of Medicine, Bethesda, USA.

7. **Appuhamy, S., Coote, J. G., Low, J. C. and Parton, R.** Identification and differentiation of isolates of *Haemophilus somnus*, *Histophilus ovis* and *Actinobacillus seminis* by PCR methods. Second European Meeting on Diagnostic PCR, The Hague, The Netherlands, 16-17, October 97. (Abstracts published in the Journal of Microbiological Methods (1997) **30:** 235-253.

8. **Appuhamy, S., Low, J. C., Parton, R. and Coote, J. G.** Specific PCR primers from the 16S-23S rRNA spacer region for the rapid detection and identification of *Actinobacillus seminis.* (submitted).

9. **Appuhamy, S., Low, J. C., Coote, J. G. and Parton, R.** PCR methods and plasmid profile analysis for characterisation of *Histophilus ovis* strains. (submitted).

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ABBREVIATIONS

A	Adenine
AFLP	Amplified restriction fragment polymorphism
AP-PCR	Arbitrarily primed PCR
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BHIA	Brain Heart Infusion Agar
BHIBYE	BHIA, blood, yeast extract
BHITTAS	BHIA, Tris, TMP (thiamine monophosphate), aspartate (sodium L) and soluble starch
C	Cytosine
CFT	Complement fixation test
CFU	Colony forming units
CNS	Central nervous system
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ERIC	Enterobacterial repetitive intergenic consensus
et al.	*et alios* (and others)
FcR	Fc receptor
G	Guanine
g	Gram
g	Gravity
h	Hour(s)
H-H	*Haemophilus-Histophilus* group
H_2O	Distilled water
H_2O_2	Hydrogen peroxide
HAP	*Haemophilus, Actinobacillus* and *Pasteurella*
HCl	Hydrochloric acid
Ig	Immunoglobulin
IPTG	Isopropyl-β-D-thio-galactopyranoside
iu	International units
kb	Kilobase(s)
KCl	Potasium chloride
KCN	Potasium cyanide
kDa	Kilodalton
MAb	Monoclonal antibody

MAT	Microagglutination test
Mb	Mega base(s)
MEE	Multilocus enzyme electrophoresis
mg	Milligram
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
µg	Microgram
µm	Micrometre
min	Minutes
ml	Millilitre(s)
mm	Millimetre (s)
Na_2HPO_4	Disodium hydrogen orthophosphate
NaCl	Sodium chloride
NCTC	National Collection of Type Cultures
ng	Nanogram
O/F	Oxidation/fermentation
°C	Degrees Celsius
OMP	Outer membrane protein
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
pH	Negative logarithm of hydrogen ion concentration
^{32}P	Phosphorous 32 isotope
pmole	Picomole
RAPD	Random amplified polymorphic DNA
REA	Restriction endonuclease analysis
REP	Repetitive extragenic palindromic elements
RFLP	Random fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription-PCR
s	Second(s)
SACVS	Scottish Agricultural College Veterinary Services
SDS	Sodium dodecyl sulphate
^{35}S	Sulphur 35 isotope
T	Thymine
TAs	Type strain of *A. seminis*
TE buffer	Tris, EDTA buffer
TEME	Thromboembolic meningoencephalitis
THs	*Haemophilus somnus* type strain

TMP	Thiamine monophosphate
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
UV	Ultraviolet
V factor	Nicotinamide adenine dinucleotide
V	Volts
v/v	Volume/volume ratio
w/v	Weight/volume ratio
X factor	Haemin
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

SUMMARY

H. somnus, *H. ovis* and *A. seminis* are closely-related Gram-negative pleomorphic bacteria. They are very similar in cultural and biochemical characteristics and these phenotypic characteristics are highly variable between strains of each species. Thus their identification is often uncertain. *H. somnus* causes thromboembolic meningoencephalitis, pneumonia and reproductive failure including abortion in cattle. *H. ovis* causes similar disease syndromes in sheep. *A. seminis* is a common cause of epididymitis and infertility in rams. Genotypic characterisation of bacteria, in many cases, has been shown to provide a clearer, more discriminating and more reliable means of differentiating species and strains within species. Polymerase chain reaction (PCR) fingerprinting methods, using different primer sets targeted against specific chromosomal regions are being applied successfully for the typing of a wide range of human and veterinary pathogens.

This work has involved the characterisation of a large number of isolates from bovine and ovine sources, including strains provided by Scottish Veterinary Investigation Centres or isolated as part of this study from slaughterhouse materials. This slaughterhouse survey showed that 18% of randomly selected reproductive tracts of cows contained *H. somnus* and this was much higher than earlier reports from elsewhere. The identity of the strains was confirmed by conventional cultural and biochemical tests (including API ZYM) and the strains were also characterised in terms of their plasmid profiles and antibiotic resistance. A high proportion of *H. ovis* isolates contained plasmids (63%) when compared to *H. somnus* (13%) and *A. seminis* (8%). Nine *H. ovis* and two *A. seminis* isolates contained two plasmids while the rest contained single plasmids. The size range of these plasmids was 1.7 kb to 5 kb. The sensitivity of these strains to a number of commonly used antibiotics was determined but there was no apparent relationship between resistance and the presence of plasmids. Although *A. seminis* is generally regarded as an ovine pathogen, one isolate was obtained from a bovine source.

The main thrust of the work was to develop PCR techniques for identification and typing. Three primer sets, namely REP, ERIC and Ribosomal, have been applied in order to generate reproducible profiles of PCR amplimers. Of the 29 *H. somnus* strains studied, 11, 16 and eight groups were recognised by the use of REP, ERIC, and Ribosomal primers respectively. For the 19 *H. ovis* isolates 11, seven and five groups were identified by the three primer sets respectively. For the 24 isolates of *A. seminis*, REP- and ERIC-PCR yielded five and nine types respectively, but PCR-ribotyping gave a similar pattern for all isolates except one. The use of a combination of the primer sets provided a high resolution fingerprinting method for these strains which could be useful for epidemiological studies of these related bacteria. PCR-ribotyping produced a relatively simple pattern which was useful for rapid identification of these species after primary isolation and for

differentiation of these species from each other and from other related bacteria. These PCR fingerprinting methods were simple to perform and reproducible. Clearly distinguishable profiles were obtained between respiratory and genital isolates of *H. somnus* by all three typing methods. The discrimination of *H. ovis* isolates was better in REP-PCR than in ERIC-PCR. In general, PCR-ribotyping produced a simple pattern but REP- and ERIC-PCR produced complex patterns. The genetic heterogeneity of *A. seminis* was revealed.

The two major amplimers of PCR-ribotyping of *A. seminis* were cloned and sequenced. The sequences showed that *A. seminis* contains at least two ribosomal operons, termed *rrnA* and *rrnB* and that these encode one and two tRNAs, respectively, in the spacer region between the 16S and 23S rRNA genes. Species-specific primers for *A. seminis* were developed from the sequence of the spacer region of *rrnB* for the specific identification and detection of *A. seminis* by PCR. The PCR assay was able to detect approximately 300 colony forming units of *A. seminis* per ml of 10-fold diluted raw semen sample. Storage solution added to semen for long-term storage was found to inhibit the PCR and the assay would best be performed for diagnostic purposes on fresh semen prior to storage.

1. INTRODUCTION

Haemophilus somnus, *Histophilus ovis* and *Actinobacillus seminis* are Gram-negative, pleomorphic bacteria. *H. somnus* and *H. ovis* cause similar clinical conditions in cattle and sheep respectively. *A. seminis* is responsible for reproductive problems of rams. They share similar cultural and biochemical properties which makes them difficult to differentiate, although historically they have been regarded as host-specific. The properties of these organisms are compared in **Table 1.1**.

1.1 *HAEMOPHILUS SOMNUS*

1.1.1 *Haemophilus somnus* - the organism

Haemophilus somnus is a Gram-negative bacterium, which causes severe disease conditions in cattle and sheep. It causes thromboembolic meningoencephalitis, pneumonia, reproductive failure, polyarthritis and mastitis resulting in severe economic losses to the cattle farming industry. The estimated economic losses due to *H. somnus* infection in cattle in Canada was over 10 million dollars during the period 1969-1978 (Saunders *et al.*, 1980). The organism and the disease have been reviewed by Bio-Ceutic Laboratories (1978), Humphrey and Stephens (1983), Miller *et al.* (1983b), Corbeil *et al.* (1986), Harris and Janzen (1989), Corbeil (1990) and Eaglesome *et al.* (1992).

1.1.1.1 History and nomenclature

Haemophilus somnus was first isolated by Kennedy *et al.* (1960), when they did detailed investigations of an outbreak of thromboembolic meningoencephalitis (TEME) in cattle. Kennedy *et al.* (1960) grouped the organism taxonomically into the genus *Haemophilus* based on similar characteristics to other members of the genus, though it is not dependent on X factor (haemin) and V factor (nicotinamide adenine dinucleotide). Later, Bailie (1969) gave it the name *Haemophilus somnus*, but the taxonomic status of the organism is not yet clear (Humphrey and Stephens, 1983). Kennedy *et al.* (1960) first described the isolates from cases of TEME as *Haemophilus*-like organisms. Subsequent isolates were described as *Actinobacillus actinoides*-like (Bailie *et al.*, 1966), *Haemophilus*-like (Case *et al.*, 1965; Panciera *et al.*, 1968) or *Actinobacillus* species (Grossling, 1966 cited by Humphrey and Stephens, 1983). On the basis of DNA composition, the requirements for growth factors and satellitism around colonies of other organisms, Bailie (1969) proposed the name *Haemophilus somnus*. The DNA composition of *H. somnus*

Table 1.1 ***Comparison of biochemical properties of H. somnus, H. ovis and A. seminis***

Property	*H. somnus*[a]	*H. ovis*[b]	*A. seminis*[a]
Catalase	--	--	+
Oxidase	+	+	D
Nitrate reduction	+	+	+
ONPG	D	N	--
Phosphatase	N	N	--
Gelatinase	N	--	N
H_2S production	N	--	N
Ornithine decarboxylase	--	N	D
Indole production	+	VR	--
Urease	--	N	--
Esculin hydrolysis	--	N	D
NAD requirement	--	N	--
X-factor requirement	--	N	N
Growth on MacConkey's agar	--	--	--
β-haemolysis, Sheep cells	--	--	--
Methyl red	N	N	N
Voges-Proskauer	N	N	--
Lysine decarboxylase	--	N	--
Arginine dihydrolase	--	N	--
α-fucosidase	N	N	N
D-adonitol (acid)	--	N	--
L-arabinose (acid)	D	--	DL
Arbutin (acid)	N	N	--
Cellobiose (acid)	--	N	--
Dextrin (acid)	N	--	--
Dulcitol (acid)	D	--	--
Meso-erythritol	--	N	N
Fructose (acid)	+	N	--
D-galactose (acid)	D	--	DL

Table 1.1 continued...

Table 1.1 continued...

Property	*H. somnus*[a]	*H. ovis*[b]	*A. seminis*[a]
D-glucose (acid)	+	+	WL
Glycerol (acid)	--	N	--
Myo-inositol (acid)	D	--	D
Inulin (acid)	--	--	--
Lactose (acid)	--	--	--
Maltose (acid)	+	--	DL
D-mannitol (acid)	+	+	DL
D-mannose (acid)	+	+	--
Melezitose (acid)	--	N	N
Melibiose (acid)	--	N	--
Raffinose (acid)	--	--	--
L-rhamnose (acid)	--	--	N
D-ribose (acid)	N	N	N
Salicin (acid)	--	--	--
D-sorbitol (acid)	+	+	--
L-sorbose (acid)	N	N	N
Starch (acid)	N	N	--
Sucrose (acid)	--	--	--
Trehalose (acid)	+	--	--
D-xylose (acid)	+	+	--
Ubiquinones present	--	N	+
Naphthoquinones present	+	N	+

[a] source: Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

[b] source: Roberts (1956); Stephens *et al.* (1983).

Symbols: +, 90% or more positive; D, 21-79% positive; --, 0-10% positive; W, weak reaction; L, delayed reaction; N, not tested; VR, variable results.

was determined by Bailie *et al.* (1973) and reported as 37.3 + 0.2 mole percentage of guanine plus cytosine. Mannheim *et al.* (1980, cited by Humphrey and Stephens, 1983) reported the DNA composition of *H. somnus* as 45.2 per cent mol% G+C.

The taxonomic status of the genera *Haemophilus, Actinobacillus* and *Pasteurella* (HAP) is under review. There has been a proposal to rank the genera as a family based on phenotypic features, DNA base composition between 38-47 mol% G+C and additional physiological and morphological criteria, with inclusion of *H. somnus* within the HAP group (Humphrey and Stephens, 1983).

1.1.1.2 Bacteriological characteristics

H. somnus is a Gram-negative, pleomorphic coccobacillus and also occurs in chains and filamentous forms. There is a reduction in the degree of pleomorphism on *in vitro* passage (Kennedy *et al.*, 1960). The organism is non-motile, non-spore-forming, non-piliated and non-acid fast (Humphrey and Stephens, 1983). The size of the bacterium ranges from 0.3-0.4 μm in width and 0.8 μm in length in coccobacilli to 0.3-0.5 μm in diameter by 1.5-4.0 μm in length in bacilli (Kennedy *et al.*, 1960). Garcia-Delgado *et al.* (1977) reported different measurements of the size of the bacterium ranging from coccoid forms of 0.7-0.9 μm in width to common coccobacillary forms of 0.8-1.1 μm in width and a predominant length of 1.2 μm. Ultrastructural studies of the organism have shown that the cell envelope is typical of Gram-negative bacteria consisting of three layers: outer membrane, inner cytoplasmic membrane and in between, a periplasmic space with a peptidoglycan layer (Stephens and Little, 1981; Humphrey and Stephens, 1983). Although the presence of a capsule was noted by Miller *et al.* (1975) and Williams *et al.* (1978), Stephens and Little, (1981) failed to demonstrate a capsule by transmission electron microscopy. Thompson and Little (1981) failed to demonstrate pili by scanning electron microscopy. van Dreumel *et al.* (1970) have reported the bipolar staining of *H. somnus*.

1.1.1.3 Cultural characteristics

Colonial morphology. Colonies of *H. somnus* grown under optimal conditions are convex, entire, moist, glistening and yellow or grey-yellow colour. Stephens *et al.* (1983) described the colonial morphology as round, pinpoint, glistening and transparent after 24 h of incubation at 37 °C in 10% (v/v) CO_2 on blood agar. After 48 h, colonies become 1-2 mm in diameter, transparent, yellowish and umbonate.

Pigmentation. Typical colonies of *H. somnus* are yellow in colour (Van Dreumel *et al.*, 1970). Williams *et al.* (1978) reported that the yellow colour is evident even when colonies are raised on a loop.

Haemolytic activity. Kennedy *et al.* (1960) observed no haemolysis on blood agar. Later, haemolytic activity of some isolates of *H. somnus* was demonstrated by Garcia-Delgado *et al.* (1977) on Brain Heart Infusion (BHI) agar containing 5% (v/v) calf blood and 0.5% (w/v) yeast extract. Humphrey *et al.* (1982a) noticed various degrees of haemolysis between strains by *H. somnus* on BHI agar supplemented with 5% (v/v) bovine blood and 0.5% (w/v) yeast extract. In contrast to this, in another study 57 (71%) of pneumonic isolates were non-haemolytic, 8 (10%) were mildly haemolytic and 15 (19%) were markedly haemolytic while 4 (20%) of genital isolates were non-haemolytic and 16 (80%) were markedly haemolytic (Fussing and Wegener, 1993).

1.1.1.4 Growth requirements

Nutritional requirements. Optimal growth was obtained on Brain Heart Infusion (BHI) agar supplemented with 0.5% (w/v) yeast extract and 10% (v/v) bovine blood (Garcia-Delgado *et al.*, 1977). Shigidi and Hoerlein (1970) used cystine heart agar with 0.5% (w/v) yeast extract and 10% (v/v) bovine blood and they found that bovine blood was better than lapine blood and much better than ovine blood. The yolk sacs of embryonated eggs have also been used for successful growth (Garcia-Delgado *et al.*, 1977). *H. somnus* is X and V factor-independent (Kennedy *et al.*, 1960; Shigidi and Hoerlein, 1970; Stephens *et al.*, 1983). Asmussen and Baugh (1981) showed a marked enhancement of growth of *H. somnus* with thiamine monophosphate or cocarboxylase (thiamine pyrophosphate) and Stephens *et al.* (1983) demonstrated the growth response of *H. somnus* to thiamine monophosphate by the disc diffusion method.

Merino and Biberstein (1982) reported that *H. somnus* had an absolute requirement for cystine or cysteine hydrochloride, but this requirement was not observed by others (Asmussen and Baugh, 1981). Stephens (1981) analysed amino acids in the media before and after growth and found no cystine or cysteine hydrochloride consumed (cited by (Humphrey and Stephens, 1983). Humphrey and Stephens (1983) suggested that cysteine hydrochloride acts to reduce the redox potential of the medium rather than as a nutrient. Amino acid analysis showed that only aspartic acid was used by *H. somnus* during growth but deliberate addition of aspartic acid was not necessary because it is abundant in most media. Addition of soluble starch also gave enhancement of growth of *H. somnus* although it was not hydrolysed. It acts as an absorbent of inhibitors which retard the growth of *H.*

somnus (Humphrey and Stephens, 1983). Stephens *et al.* (1983) have used 5.7% (w/v) BHI agar (although the manufacturer's recommended concentration of BHIA is 4.7% (w/v)), 0.05% (w/v) Na-L-aspartate, 0.1% (w/v) soluble potato starch, 0.1% (w/v) Tris and 0.001% (w/v) thiamine monophosphate as a blood free solid medium and 3.2% (w/v) BHI (rather than the manufacturer's recommended concentration of 3.7% (w/v)), with the other ingredients as a broth medium. For fermentation tests, 1.0% (w/v) proteose peptone, 0.05% (w/v) Na-L-aspartate, 0.1% (w/v) soluble potato starch, 0.1% (w/v) Tris, 0.2% (w/v) Na_2HPO_4 (anhydrous), 0.5% (w/v) NaCl, 0.2% (w/v) carbohydrate, 0.001% (w/v) thiamine monophosphate and 0.05% (w/v) L-cysteine hydrochloride is suitable (Stephens *et al.*, 1983). Inzana and Corbeil (1987) developed a defined medium for the growth of *H. somnus*. They reported that components required for or enhancing the growth of most *H. somnus* isolates in the defined medium included uracil, D-glucose, isotonic NaCl, Na_2HPO_4, flavin mononucleotide, pantothenic acid, pyridoxine and salts and amino acids.

CO_2 requirement. Primary isolation of *H. somnus* requires 5-10% (v/v) CO_2 and subsequent growth was successful in aerobic conditions (Kennedy *et al.*, 1960; Van Dreumel *et al.*, 1970; Garcia-Delgado *et al.*, 1977). Shigidi and Hoerlein (1970) reported little difference in growth in atmospheres containing 5%, 10% or 20% (v/v) CO_2 and Canto and Biberstein (1982) reported that strains of *H. somnus* grew in ambient air. Although Stephens *et al.* (1983) stressed that *H. somnus* grows only in air containing 10% (v/v) CO_2, Inzana and Corbeil (1987) reported that *H. somnus* grew best in 10% (v/v) CO_2 but variants developed that adapted to growth in air. They obtained the highest density in the shortest time under maximum aeration. They also suggested that the requirement for CO_2 was as a nutritional factor rather than by making the environment less aerobic.

Temperature. Maximum growth was obtained by Shigidi and Hoerlein (1970) and Garcia-Delgado *et al.* (1977) at 37 °C but the former also observed moderate growth at 30 °C and 43 °C but no growth at 27 °C and 47 °C.

pH. Maximum growth was obtained at pH 7.8 in an atmosphere containing CO_2 (The percentage of CO_2 was not mentioned) (Shigidi and Hoerlein, 1970).

1.1.1.5 Biochemical characteristics

The reported biochemical characteristics are highly variable. Garcia-Delgado *et al.* (1977) mentioned that the variability may be due to variation in techniques used rather than due to differences in the organisms themselves. The biochemical and fermentation

characteristics of *H. somnus* reported by various investigators are summarised in **Tables 1.2** and **1.3**.

1.1.1.6 Storage of *H. somnus* isolates

Initially, *H. somnus* isolates were stored in egg yolk at -70 °C (Garcia-Delgado *et al.*, 1977) and isolates have readily been recovered from this medium after eight years (Humphrey and Stephens, 1983). More recently, other media have been reprorted such as 60% glycerol in phosphate buffered saline at -70 °C (Widders *et al.*, 1989a), 3.7% BHI, 0.05% sodium acetate, 0.1% soluble potato starch, 0.1% Tris and 0.001% thiamine monophosphate (w/v) supplemented with 10% glycerol (Fussing and Wegener, 1993) and tryptic soy broth with 10% glycerol (Salmon *et al.*, 1993).

1.1.1.7 Selective media for *H. somnus*

A selective medium has been developed for isolation of *H. somnus* from cattle and sheep incorporating vancomycin (5 µg/ml), neomycin (5 µg/ml), sodium azide (50 µg/ml), nystatin (100 iu/ml) and cycloheximide (100 µg/ml) into 5% horse blood agar with TMP (1 µg/ml) (Slee and Stephens, 1985). The authors reported that Gram-positive bacteria did not grow on this selective medium and many Gram-negative bacteria were also inhibited. They also reported that the selective medium was better than sheep blood agar and was stable for two weeks at 4 °C. However, Kwiecian and Little (1989) reported that this selective medium lowered the isolation rate of *H. somnus*. In reply, Slee and Stephens (1985) reasoned that this failure was due to two reasons: first the medium was not duplicated correctly and second that a difference in CO_2 percentage in the incubator would change the pH of the medium. Brewer *et al.* (1985) also developed media for transport and isolation of *H. somnus*. They used BHI broth supplemented with 0.5% yeast extract, 10% foetal calf serum and antibiotics lincomycin (3 µg/ml) and cycloheximide (100 µg/ml) as a transport medium. A selective medium containing BHIA supplemented with 0.5% yeast extract, 5% sheep blood, 5% horse serum and the antibiotics lincomycin (3 µg/ml) and cycloheximide (100 µg/ml) facilitated the isolation of *H. somnus* from contaminated materials. None of these selective media prevented the growth of *Proteus* (Brewer *et al.*, 1985; Slee and Stephens, 1985). Brewer *et al.* (1986) modified the original medium of Brewer *et al.* (1985) and found a medium containing lincomycin (3 µg/ml), cycloheximide (100 µg/ml) and chloral hydrate (0.1%) was superior for isolation of *H. somnus* from materials contaminated with *Proteus* species.

Table 1.2 ***Biochemical properties of H. somnus***

Test	Ref 1	Ref 2	Ref 3	Ref 4	Ref 5	Ref 6	Ref 7	Ref 8	Ref 9
Catalase		--		--	--	--		--	--
Citrate utilisation				--		--	--	--	
Gelatin liquefaction	--	--				--			
Growth on MacConkey agar	--							--	
H_2S production	--	--		+		+	--	+	
Indole	**WP**	(+)	--	(+)	(+)	(+)	**V**	(+)	+
Lecithinase					--				
Litmus milk	**WP**	--				+			
Nitrate reduction	**WP**		--	+	+	+		+	+
Oxidase		--		+	+	+	+	+	+
Urease		--		--	--	--	--	--	--

+	positive	Ref 1	Kennedy *et al.* (1960)
(+)	majority positive	Ref 2*	Gossling (1966)
--	negative	Ref 3*	Panciera *et al.* (1968)
WP	weak positive reaction	Ref 4*	Bailie (1969)
V	variable reactions	Ref 5	Shigidi and Hoerlein (1970)
		Ref 6*	Corboz and Nicolet (1975)
		Ref 7	Garcia-Delgado *et al.* (1977)
		Ref 8	Stephens *et al.* (1983)
		Ref 9	Holt *et al.* (1994)

*cited by Humphrey and Stephens, (1983)

Table 1.3 ***Fermentation properties of H. somnus***

Sugar	Ref 1	Ref 2	Ref 3	Ref 4	Ref 5	Ref 6	Ref 7
arabinose	+	V	V	V	V	V	(+)
dulcitol	(--)	V	V	V	V		(+)
fructose	--						+
galactose					--		(+)
glucose	+	+		+	+	+	+
inositol	--	V	V	V	V		(+)
lactose	--	V	V	V	V	V	--
levulose		(+)		(+)	(+)		
maltose	+	(+)		(+)	(+)		+
mannitol	--				(+)	V	+
mannose	+	(+)		(+)	(+)		+
raffinose	--	V	V	V	V		--
rhamnose	+	V	V	V	V		--
salicin						--	--
sorbitol	+				(+)	V	+
sucrose	(--)				--	--	--
trehalose		(+)		(+)	(+)	V	+
xylose	+	(+)		(+)	(+)	V	+

+	positive	Ref 1	Kennedy *et al.* (1960)
(+)	majority positive	Ref 2*	Gossling (1966)
--	negative	Ref 3*	Panciera *et al.* (1968)
(--)	majority negative	Ref 4*	Bailie (1969)
V	variable reactions	Ref 5*	Corboz and Nicolet (1975)
		Ref 6	Fussing and Wegener (1993)
		Ref 7	Holt *et al.* (1994)

*cited by Humphrey and Stephens, (1983)

1.1.1.8 Antimicrobial sensitivity

H. somnus is found to be highly susceptible to most common antimicrobial agents, using disc diffusion tests in different media (Kennedy *et al.*, 1960; Van Dreumel *et al.*, 1970; Garcia-Delgado *et al.*, 1977). These authors have also reported that some *H. somnus* isolates have shown resistance in different occasions to chlortetracycline, bacitracin, lincomycin, sulphonamides, neomycin, oxacillin, spiramycin, polymyxin B, chloramphenicol, penicillin and streptomycin. The minimum inhibitory concentrations (μg/ml) of different antibacterial drugs for *H. somnus* were as follows: amoxycillin-clavulinic acid, 0.08; polymyxin B, 1.6; colistin, ≤0.1; streptomycin, 6.3; neomycin, 25; kanamycin, 6.3; gentamicin, 6.3; spectinomycin, 25; erythromycin, 1.6; tylosin, 6.3; spiramycin, 100; tilmicosin, 6.2; tiamulin, 1.6; chloramphenicol, 0.8; tetracycline, 1.6; sulphonamides, ≥100; ciprofloxacin, 0.015; enrofloxacin, 0.015; norfloxacin, 0.125; novobiocin, ≤0.1 (Prescott and Baggot, 1993).

Sugimoto *et al.* (1983) tested 33 antimicrobial agents against *H. somnus* for sensitivity using minimal inhibitory concentrations. A marked susceptibility was shown to penicillin G, ampicillin, colistin and novobiocin and resistance to spiramycin and sulphadimethoxine. Some isolates were resistant to streptomycin. Slee and Stephens (1985) reported that *H. somnus* isolates were sensitive to cephalothin, sulphonamides and trimethoprim and resistant to sodium azide, cycloheximide, neomycin, nystatin and vancomycin. They explained that media containing para amino benzoic acid are not suitable for testing sulphonamide susceptibility (Slee and Stephens, 1985).

1.1.1.9 Plasmids

The term plasmid was originally used to describe all extrachromosomal hereditary determinants. At present, the term is used only for extrachromosomal DNA which replicates autonomously. The size of plasmids ranges from one to 200 kb or even more. Although plasmids are not essential for the survival of bacteria under all conditions, they can carry important genetic determinants which permit the survival of the bacterium in adverse environmental conditions. The medical importance of plasmids is that they may carry the genetic material for resistance to antibiotics and for virulence factors such as toxins and aggressins. There are other useful properties of plasmids too, for example in biotechnology and especially in molecular cloning (Brown, 1986).

Fussing and Wegener (1993) screened Danish bovine isolates of *H. somnus* and found plasmids in 20% of isolates. Among them, 15% of pneumonic isolates and 40% of

genital isolates contained plasmids. Five percent of isolates possessed two plasmids and 1% yielded three plasmids. The size of the plasmids ranged from 1.5-3.5 kb. The significance of these plasmids has not been determined.

1.1.1.11 Antigens of *H. somnus*

Early work suggested that the majority of *H. somnus* isolates were antigenically identical (Shigidi and Hoerlein, 1970; Garcia-Delgado *et al.*, 1977). Later, Canto and Biberstein (1982) showed serological diversity in *H. somnus* using cross adsorption agglutination tests with isolates from North America and from Europe. They demonstrated a common antigen (antigen C) in all isolates, an antigen from American isolates (antigen A) and an antigen from European (Swiss) isolates (antigen S). Molecular and antigenic differences between smooth and mucoid colonial variants of *H. somnus* have been demonstrated (Humphrey and Stephens, 1983). Both contain common 53, 36.2 and 25.6 kDa proteins. But the smooth variants also had proteins of 16, 14.8 and 11.7 kDa whereas mucoid variants had proteins of 13.8, 12.1 and 11.5 kDa. The 36.2 and 25.6 kDa components were serologically similar, indicating that they were common antigens, while serological differences were observed in the lower molecular weight fractions, indicating that the variants possessed specific antigens.

1.1.1.12 Serological cross-reactivity with other bacteria

Several investigators have shown that some bacterial species cross-react with *H. somnus* in bacterial agglutination tests. They are *H. agni* (Kennedy *et al.*, 1960; Shigidi and Hoerlein, 1970; Miller *et al.*, 1975), *A. lignieresii* (Miller *et al.*, 1975), *Campylobacter fetus* (Miller *et al.*, 1975), *Bordetella bronchiseptica* (Shigidi and Hoerlein, 1970), *Moraxella bovis* (Garcia-Delgado *et al.*, 1977), *Yersinia enterocolitica* (Garcia-Delgado *et al.*, 1977) and *Streptococcus agalactiae* (Miller *et al.*, 1975). However, no cross-reactivity between *H. somnus* and *B. bronchiseptica* was found by Miller *et al.*(1975) and Garcia-Delgado *et al.* (1977), or between *H. somnus* and *A. actinoides* (Shigidi and Hoerlein, 1970; Miller *et al.*, 1975), *Pasteurella haemolytica, P. multocida, A. equuli, Neisseria catarrhalis, Brucella abortus* (Garcia-Delgado *et al.*, 1977), *E. coli, Salmonella dublin* or *Staphylococcus aureus* (Miller *et al.*, 1975).

Using complement-fixation tests, Dierks *et al.* (1973) showed a weak serological relationship between *H. somnus* and *H. agni, H. aegypticus, H. aphrophilus, H. influenzae, H. parainfluenzae, A. actinoides* and *Mycobacterium bovis*. Miller *et al.* (1975)

found weak cross reactions between *H. somnus* and *A. actinoides, A. lignieresii, B. bronchiseptica, B. abortus, E. coli, H. agni, Listeria monocytogenes, S. dublin, Streptococcus agalactiae* and *Corynebacterium* species by passive haemagglutination tests.

1.1.2 Diseases caused by *Haemophilus somnus*

1.1.2.1 Epidemiology

Thromboembolic meningoencephalitis (TEME) was first recorded in Colorado in the USA by Griner *et al.* (1956) but they did not isolate the causative agent. In 1960, Kennedy *et al.* (1960) isolated the causative agent *H. somnus* from an outbreak of TEME in cattle in Colorado, USA. Later reports were published on the disease in the United States (Shigidi and Hoerlein, 1970; Smith and Biberstein, 1977), in Canada, (Van Dreumel *et al.*, 1970; Saunders and Janzen, 1980; Saunders *et al.*, 1980). The disease is now spreading in notherly and easterly directions including Russia, Germany, Switzerland, Italy, the United Kingdom, Romania and Japan (Humphrey and Stephens, 1983).

Although primarily a disease of feedlot cattle (Kennedy *et al.*, 1960; Saunders *et al.*, 1980), TEME may affect dairy animals (Saunders *et al.*, 1980) and cattle on pasture (Humphrey and Stephens, 1983). TEME is most prevalent in the autumn and winter. The predisposing factors for TEME are introduction of calves to feedlots, transport of animals and other stress factors. Most cases of TEME are reported at the age of seven to nine months (Saunders *et al.*, 1980) but animals may be affected at less than four months to more than 24 months (Humphrey and Stephens, 1983). Saunders *et al.* (1980) also reported that cases of *H. somnus*-associated respiratory disease were more prevalent in spring and summer and relatively more cases were reported in dairy calves than beef calves. Donkersgoed *et al.* (1990) found that Haemophilosis mainly occurred three to five weeks after arrival in the feedlot and the incubation period of infection may be as short as two days or as long as 21 days, depending on stress and the immune status of the infected calf. Low titres of antibody to *H. somnus* in some calves could reflect a low exposure rate to *H. somnus* or failure of passive transfer of antibody in these calves (Donkersgoed *et al.*, 1993).

Environmental survival of *H. somnus* in different biological fluids and at different temperatures was determined by Dewey and Little (1984). They found that survival of the organism beyond 70 days occurred when it was mixed with cerebrospinal fluid, whole blood, blood plasma, vaginal mucus at -70 °C. Survival in these biological fluids was less than five days at 3 °C. At 23.5 °C the organism survived beyond 70 days when mixed with

blood and nasal mucus. At 37 °C, survival was demonstrated for more than 70 days in whole blood and blood plasma. In urine, the viability of *H. somnus* was less than one day at any of the temperatures tested.

Harris and Janzen (1989) observed that in past years, TEME cases were common. More recently, however other forms of disease due to *H. somnus* i.e. myocarditis, arthritis, myelitis have become more common. They suggested two possible reasons for their observations. First, proper herd management by early diagnosis and treatment, vaccination and mass medication may alter the expression of virulence of *H. somnus* resulting in subacute forms of the disease. Secondly, less virulent strains may be evolving by natural selection.

1.1.2.2 Carrier states of *H. somnus*

Respiratory carrier state. *H. somnus* has been isolated from nasal cavities (Saunders and Janzen, 1980) and tracheas of clinically normal cattle. An increased isolation rate is found in animals in which TEME has occurred. Gogolewski *et al.* (1989) demonstrated that experimental chronic *H. somnus* pneumonia in calves followed a three day course and bacteria persisted in the lung for six to ten weeks or more. *H. somnus* was recovered by bronchoalveolar lavage when nasal cultures were negative.

Uro-genital carrier state. The reproductive tract is usually considered to be the ecological niche or reservoir of *H. somnus* (Harris and Janzen, 1989). There is a high prevalence of *H. somnus* in the male bovine reproductive tract (60%) and semen (50%) of clinically normal bulls (Humphrey *et al.*, 1982b) and *H. somnus* was isolated from 77% of reproductive tracts of bulls in a slaughter house survey (Humphrey *et al.*, 1982a). A known encephalopathic strain of *H. somnus* was found to establish readily in the prepuce of a bull without loss of virulence, confirming that the prepuce will support growth and survival *H. somnus* (Humphrey *et al.*, 1982a).

Miller *et al.* (1983a) reported that 8% of genital tracts of cows contained *H. somnus*. It was isolated from vagina, uterus and major vestibular glands. They also reported no correlation between isolation of *H. somnus* and inflammatory lesions in the reproductive tract. The isolation of *H. somnus* from cervical mucus is increased in the post partum period and non venereal spread from cow to calf during neonatal period has been reported (Humphrey & Stephens, 1983). Urinary excretion has been described as one method of environmental contamination with *H. somnus* and may represent a significant factor in its transmission (Saunders *et al.*, 1980; Stephens *et al.*, 1981; Humphrey and

Stephens, 1983). Kaneene *et al.* (1987) showed that *H. somnus* is transient in the bovine uterus. In a slaughterhouse survey, Kwiecien and Little (1992) isolated *H. somnus* from uterus and cervix, in 7.9% and 2.7% of animals respectively. *H. somnus* has also been isolated from bovine milk (Greer *et al.*, 1989).

1.1.2.3 Diseases

The disease syndromes associated with *H. somnus* have been well reported. It affects brain, respiratory tract, reproductive tract, joints, heart and mammary gland predominantly in cattle, causing TEME, pneumonia, reproductive failure, arthritis, myocarditis and mastitis respectively.

Thromboembolic meningoencephalitis (TEME) - Peracute form. TEME was first described by Griner *et al.* (1956) and was associated with clinical symptoms such as sudden death, blindness, incoordination, depression, convulsions and occasional excitement and irritability. One to three years old cattle were commonly affected and older and young animals were also affected. The rectal temperature ranged from 99 °F to 107 °F with the stage of the infection (Griner *et al.*, 1956). Kennedy *et al.* (1960) reported the clinical signs of TEME as sudden death, stupor, opisthotonus, ataxia, weakness and paralysis. The neurological signs are highly variable and invariably lead to death (Kennedy *et al.*, 1960). The other neurological signs are muscle tremor, irritability, knuckling, limb stiffness, reluctance to move and paddling.

In animals with more protracted disease, musculoskeletal disturbances including knuckling at fetlock, stiffness, reluctance to move, single or multiple leg lameness, arthritis and extended or elevated attitude of the head may be observed. The other signs associated with TEME include tachycardia, hyperpnoea, anorexia and normal to subnormal body temperature in the terminal stage (Humphrey and Stephens, 1983). Harris and Janzen (1989) reported that TEME is a sequel of the septicaemic form of disease caused by virulent organisms. Affected animals were usually calves between six and ten months of age which had arrived at the feedlot three to four weeks previously. The retina of the affected animals show scattered haemorrhages with ill-defined borders and it is a diagnostic feature when present (Stephens *et al.*, 1981).

Humphrey and Stephens (1983) have reported that up to 90% of animals affected with TEME previously suffered from the respiratory form of the disease, with symptoms of dry harsh cough, increased respiratory rate, mild depression and elevated body

temperature. If the respiratory form did not progress to TEME, the symptoms subsided after a few days.

Respiratory form. Respiratory disease caused by *H. somnus* is characterised by fibrinous pneumonia and pleuritis without involvement of the central nervous system (CNS) (Saunders *et al.*, 1980). Commonly, respiratory infection is the first sign of *H. somnus* infection. However, in some instances, the septicaemic form of the disease or CNS involvement occurs without prior signs of respiratory disease. The other signs are dyspnea, nasal discharge, depression and elevated body temperature to 41.1 ^{o}C. There have been reports of sudden death in calves with pneumonia from which *H. somnus* was isolated with few pulmonary signs. Humphrey and Stephens (1983) reported that *H. somnus* should be recognised as an aetiological agent of acute fibrinous pneumonia, a cause of sudden death in calves.

The site of entry of *H. somnus* is thought to be the respiratory tract and *H. somnus* causes both upper and lower respiratory tract infections. In the upper respiratory tract, *H. somnus* causes laryngitis and tracheitis. In most pneumonic cases, *H. somnus* is the only bacterium isolated but sometimes *Pasteurella, Actinomyces, Fusobacterium* and *Clostridium* species have been present as secondary opportunists (Harris and Janzen, 1989). There are many reports claiming that lower respiratory tract infection often proceeds to TEME (Saunders *et al.*, 1980; Humphrey and Stephens, 1983; Harris and Janzen, 1989). In case control studies, *H. somnus* has been found in pure culture in as many as 28% of cases of pneumonic lungs examined microbiologically (Groom, 1985, cited by Harris and Janzen, 1989). *H. somnus* usually causes bronchopneumonia and it is also involved as a part of the shipping fever complex. In some cases of pneumonic pasteurellosis, *H. somnus* may have been the primary cause but its isolation is prevented because of the more rapid growing *Pasteurella* organisms or antimicrobial therapy.

H. somnus is one of a number of common causative agents of Bovine Respiratory Disease complex which is common in feedlot calves 6-8 months of age. The mean day of onset for fatal pneumonia is 12 days after animals arrive in the lot and day 22 is the mean day of onset for myocarditis and pleuritis. The *H. somnus* form of this complex is characterised by toxaemic suppurative pneumonia, pleuritis and persistent fever for several days and sudden deaths due to myocarditis (Radostits *et al.*, 1994).

Urogenital form. In males, *H. somnus* is commonly isolated from preputial washings, and also from the bladder, accessory sex glands and ampullae (Humphrey *et al.*, 1982a; Humphrey *et al.*, 1982b; Harris and Janzen, 1989). The clinical significance or pathogenic role of *H. somnus* in the bull's reproductive tract is not clear (Harris and

Janzen, 1989). Janzen *et al.* (1981) reported that 47% of semen samples from bulls were positive for *H. somnus* and the age of affected bulls ranged from one to ten years, being highest at three to four years. *H. somnus* was found in 50% of unprocessed bovine semen samples in one study (Humphrey *et al.*, 1982a). Metz *et al.* (1984) diagnosed chronic suppurative orchiepididymitis in a calf associated with *H. somnus*. Except for a few reports of infertility and poor semen quality, the organism does not seem to cause disease in these sites (Harris and Janzen, 1989).

In females, the organism has been isolated from both fertile and infertile cows (Kwiecien and Little, 1991) although *H. somnus* is a known causative agent of infertility in cows. *H. somnus* is a major cause of endometritis and it has been isolated from infertile cows with metritis and endometritis (Humphrey and Stephens, 1983). van Dreumel & Kierstead (1975) isolated the organism from mucopurulent discharge of aborted cows. Patterson *et al.* (1984) observed severe mucopurulent discharge with vaginitis and cervicitis a week after introduction of *H. somnus* carrier-bulls into a herd of heifers for mating. They reported an 82% fertility rate in that study. Kannene *et al.* (1986a; 1986b) showed *H. somnus* causes the degeneration of early embryos and also it reduces the *in vitro* survival time of embryos from *H. somnus*-infected heifers compared to those from control heifers. They suggested that the embryonic death may be due to a direct effect on the embryo by altering the local conditions of the uterus presumbly because of bacterial growth and presence of toxins rather than the direct effect of endometritis. Kannene *et al.* (1987) reported that there may be a link between the development of cystic ovarian disease and intrauterine inoculation of *H. somnus*. However, it requires extended experiments for confirmation (Kwiecien and Little, 1991).

There are several reports showing that *H. somnus* is an abortificient agent (Chladek, 1975; Van Dreumel and Kierstead, 1975; Saunders *et al.*, 1980; Humphrey and Stephens, 1983) and the organism has been isolated from aborted fetal tissue or stomach contents (Corbeil *et al.*, 1986). Abortions are sporadic rather than abortion storms and occur at any stage of gestation. Abortions have been induced experimentally by both intrabronchial and intravenous inoculation of *H. somnus* and the organism has been isolated from foetal stomach contents, placenta, uterus, cervix and vagina from experimentally aborted cows. No pathological lesions in foetuses have been reported but suppurative necrotizing placentitis and retention of the placenta are constant features (Corbeil *et al.*, 1986). *H. somnus* also causes weak calf syndrome and is characterized by abortion, stillbirth, neonatal death and the birth of weak calves with impaired body function associated with *H. somnus* infection (Waldhalm *et al.*, 1974).

Other diseases. *H. somnus* causes a peracute form of mastitis with systemic signs in dairy cattle (Armstrong *et al.*, 1986). Chronic and gangrenous mastitis have been produced experimentally (Hazlett *et al.*, 1983; Grinberg *et al.*, 1993). The changes of the milk vary from blood-tinged and watery with small fibrin clots to white homogeneous without evidence of blood (Harris and Janzen, 1989).

H. somnus also affects joints resulting in polyarthritis which is characterized by firm swelling of joints and has been observed two days to several weeks following TEME outbreaks. Multiple joints are usually affected and the animals show lameness, stiffness and knuckling at the fetlock (Panciera *et al.*, 1968, cited by Harris and Janzen, 1989).

H. somnus has been isolated from the diseased conjunctival sac of cattle with corneal opacity (Lamont and Hunt, 1982) but the authors suggested that the isolation was not sufficient evidence that the lesions were caused by this organism.

1.1.2.4 Experimental infection

Stuart *et al.* (1990) reported that *H. somnus* could cause placentitis and abortion in cattle after intravenous inoculation but this most probably occurs in the presence of pre-existing placental damage.

Stephens *et al.* (1981) inoculated cattle with *H. somnus* intravenously resulting in 70% mortality from TEME. *H. somnus* was isolated more frequently and in greater numbers from the CNS and urinary tract than from other organs.

Differences in virulence of three strains of *H. somnus* from different anatomical sites have been shown by Groom *et al.* (1988) by induction of experimental pneumonia in calves after intratracheal inoculation. Potgieter *et al.* (1988) induced *H. somnus* respiratory disease experimentally in four to six months old calves by intrabronchial inoculation. The pneumonic lesions were similar to those described for naturally occurring *H. somnus*-associated respiratory disease such as necrotising, suppurative, lobular bronchopneumonia and pleuritis.

1.1.2.5 Virulence factors of *H. somnus*

Molecular aspects of some of the virulence factors of *H. somnus* have been reviewed by Corbeil (1990). Pathogens differ from non-pathogens by producing some

factors which contribute to the disease process. One important observation on *H. somnus* is that isolates may be both pathogenic and non pathogenic. Humphrey and Stephens (1983) and Humphrey *et al.* (1982b) have reported that normal bulls carry *H. somnus* on the preputial epithelium and normal cows or heifers carry the organisms on the vaginal mucosae. TEME (Stephens *et al.*, 1981; Stephens *et al.*, 1982), pneumonia (Jackson *et al.*, 1987; Gogolewski *et al.*, 1987a; Groom *et al.*, 1988) and abortions (Miller *et al.*, 1983c; Widders *et al.*, 1986) have been reproduced experimentally with pure cultures of *H. somnus* from diseased and normal animals.

The virulence factors will be described according to the sequence of events involved in causing disease. These events are the attachment of the bacteria to the host, invasion through the skin or mucous membranes, multiplication in the host, interference with host defence mechanisms and damage to host cells or tissues resulting in the disease.

H. somnus first establishes itself in the host by colonizing the surface of the mucous membranes and then attaching to non-epithelial cells (Corbeil, 1990). Thompson and Little (1981) first showed that *H. somnus* isolates from both an aborted foetus and from fatal TEME became adherent to endothelial cells in cultures of carotid artery segments. Bacteria of the abortion strain adhered to a greater extent than those of the TEME strain. *H. somnus* was also internalized by phagocytosis into phagocytic vacuoles or in endothelial cell membrane folds. Kwiecien *et al.* (1994) have demonstrated that various strains of *H. somnus* adhere to bovine aortic endothelial cells in culture and that adherence is enhanced by tumour necrosis factor-α, but adherence does not correlate with pathogenicity. They suggested that intrinsic adhesive properties of *H. somnus* to bovine aortic endothelial cells do not explain clinical pathogenicity, but provide evidence that tumour necrosis factor-α might enhance bacterial adhesion to vascular endothelium if infection is established. The attachment of *H. somnus* to capillary endothelium may be related to the pathogenesis of vasculitis and thrombosis which are cardinal characters of *H. somnus* causing TEME, pneumonia and abortion (Humphrey and Stephens, 1983; Widders *et al.*, 1986). Kaneene *et al.* (1986a) have suggested that *H. somnus* may induce early embryonic degeneration by creating an unfavorable uterine environment for the embryos and that may be due to production of a toxin. Kaneene *et al.* (1987) showed that *H. somnus* may have a short and long term effect on ovarian activity by inducing cystic follicles.

H. somnus has also been shown to attach to the cultured cells of a bovine turbinate cell line (Ward *et al.*, 1984) and primary bovine vaginal epithelial cells (Corbeil, 1990). Thomson *et al.* (1988) demonstrated *H. somnus* adhered to 10 of 41 zona pellucida of intact bovine embryos. The adherence to mucus and cell surfaces may be important in pathogenesis but the mechanism is not clear (Corbeil, 1990). Stephens and Little (1981)

were unable to demonstrate pili with negative staining or a capsule with ruthenium red staining.

In the asymptomatic carrier state, *H. somnus* remains at the mucosal surface without invading cells. Although the mechanism of invasion is not clear, the invasion must occur to produce septicaemia and its sequelae i.e. TEME, arthritis, haematogenous abortion. It is clear that to survive in the blood stream it is necessary for the bacterium to be resistant to complement-mediated killing (Corbeil, 1990). Although Simonson and Maheswaran (1982) reported the antibacterial activity of fresh bovine serum, Corbeil *et al.* (1985a) showed that *H. somnus* isolates from diseased animals were serum resistant and some isolates from preputial carriers were serum susceptible.

The factors responsible for *H. somnus* multiplication *in vivo* are not defined. Although the competition with the host for nutrients is probably a major factor, little has been revealed in this area. However, it has been shown that the normal microflora plays a major role in the multiplication of *H. somnus*. By cross-streaking techniques, Corbeil *et al.* (1985b) demonstrated that most isolates of the normal bacterial flora influence the growth of *H. somnus* with enhancers outnumbering inhibitors by about four to one. Thus the ratio of enhancers to inhibitors *in vivo* could affect multiplication of *H. somnus*.

Interference with host defence mechanisms by *H. somnus* has been studied by several investigators. Although bovine neutrophils readily ingest *H. somnus*, they are unable to kill the organism *in vitro* and perhaps bacteria multiply within bovine neutrophils (Czuprynski and Hamilton, 1985a). Light and electron microscopic examination of *H. somnus*-infected monocytes revealed the intracellular growth of the bacterium and this process presumably contributes to the subacute and chronic course of the disease (Lederer *et al.*, 1987). Bovine neutrophils or blood monocytes (Czuprynski and Hamilton, 1985b) or alveolar macrophages (Lederer *et al.*, 1987) do not kill *H. somnus*. Sample and Czuprynski (1991) showed that *H. somnus* removes H_2O_2 from solution by an energy-dependent process. They suggested that this ability of *H. somnus* may be an important virulence mechanism that contributes to the survival of the organism following ingestion by bovine neutrophils. Pfeifer *et al.* (1992) evaluated the effect of *H. somnus* on polymorphonuclear leukocyte function using a flow cytometric technique, nitroblue tetrazolium and chemiluminescence assays to determine the polymorph respiratory burst. The *in vitro* exposure of polymorphonuclear leukocytes to logarithmically growing *H. somnus* reduced the respiratory burst of polymorphonuclear leukocytes from healthy calves. Polymorphs from calves with acute *H. somnus* disease also showed reduced respiratory burst activity. This modulation of bovine polymorph function by *H. somnus* may thus contribute towards disease pathogenesis. Hubbard *et al.* (1986) showed that

intracellular fractions of *H. somnus* suppressed iodination of protein by bovine polymorphs and two fractions of high (>300 kDa) and low (<1 kDa) molecular weight were involved. These suppressive factors were released into the surrounding medium during growth of the bacterium and were identified as adenine, guanine and guanine monophosphate (Chiang *et al.*, 1986). The host would not normally produce antibodies against these low molecular weight molecules (Corbeil, 1990).

Czuprynski and Hamilton (1985a) found that in the absence of antibody to *H. somnus*, ingestion by neutrophils did not occur but in the presence of antibody ingestion occurred without killing of the bacterium. Gogolewski *et al.* (1989) showed differences in immunoglobulins in serum and bronchoalveolar lavage after experimental chronic *H. somnus* pneumonia. In bronchoalveolar secretions IgG1, IgG2, IgM and IgA antibodies specific for *H. somnus* were detected. In serum, IgG1, IgG2 and IgM but no IgA were detected, suggesting that both local and systemic antibody responses had occurred.

By electron microscopy, it was shown that *H. somnus* multiplies within bovine mononuclear leucocytes, and later these leucocytes degenerated (Lederer *et al.*, 1987). The same findings were made by Gogolewski *et al.* (1987a) and alveolar macrophage degeneration is a characteristic feature of *H. somnus* intrabronchial challenge in young calves. *H. somnus* causes damage to vascular endothelium (Thompson and Little, 1981) and alveolar macrophages (Gogolewski *et al.*, 1987a) and the damage to both type of cells has been demonstrated *in vitro* (Lederer *et al.*, 1987) and *in vivo* (Gogolewski *et al.*, 1987a; Gogolewski *et al.*, 1987b).

Although Lederer *et al.* (1987) showed that *H. somnus* is a facultative intracellular pathogen of bovine mononuclear phagocytes, Corbeil (1990) concluded that *H. somnus* is predominantly extracellular with *in vivo* studies. Silva and Little (1990) have suggested that serum factors other than humoral antibodies are involved in the resistance of cattle against *H. somnus* infection. Nevertheless, by passive protection tests, Gogolewski *et al.* (1987a) have shown protection by specific antibody. The predominant reaction observed with convalescent serum was with a 78 kDa outer membrane protein (OMP) and a 40 kDa OMP in Western blots (Corbeil, 1990). Gogolewski *et al.* (1988) also showed that IgG1 was the antibody specific for the 78 kDa OMP and it was not protective but, for the 40 kDa OMP, both specific IgG1 and IgG2 were present and they were protective. Thus, the 40 kDa antigen could be considered as an important virulence factor (Corbeil, 1990). Corbeil *et al.* (1991) have characterized the 40 kDa antigen of *H. somnus* as two distinct antigens: One of 40 kDa was common to the family *Pasteurellaceae* and the other of 39 kDa only cross-reacted with *Haemophilus agni*, suggesting that it might be a good immunodiagnostic antigen.

Wedderkopp *et al.* (1993) have investigated the differences in protein expression of *H. somnus* in iron-restricted growth conditions. They found new outer membrane proteins were expressed under iron-restricted growth conditions and that there were differences among strains in the number of induced proteins and in their molecular weights, but there was no specific relationship between these strain-dependent differences and tissue trophism.

The Fc receptor (FcR) activity of *H. somnus* was first described by Widders *et al.*, (1988). The Fc receptors are proteins that are present in the outer membrane of *H. somnus* and bind with the Fc portion of bovine immunoglobulin non-immunely and are probably involved in serum resistance (Corbeil, 1990). There are two types of FcRs, one of 41 kDa which binds weakly with IgG1, IgG2, IgM and IgA and three high molecular weight receptors of 350, 270 and 120 kDa which bind strongly with IgG2, IgM and IgA. All four receptors are antigenically related and the 41 kDa receptor appears to be a subunit of the higher molecular weight receptors (Yarnall *et al.*, 1988a). The latter are secreted into the culture medium and are also in the insoluble protein fraction of the culture supernatant. The 41 kDa protein that is a major OMP, is only present in the insoluble protein fraction of culture supernatant (Yarnall *et al.*, 1988b). In relation to the significance of FcR as a virulence factor, Widders *et al.* (1989a) showed an association between the presence of FcR on the surface of *H. somnus* and serum resistance. They found that the isolates from diseased animals and vaginal carrier isolates bound to IgG Fc fragments whereas isolates from preputial carriers did not show binding activity. The low-binding isolates lacked both the 270 and 41 kDa receptor proteins whereas high binding isolates had both receptors. The high binding isolates were serum resistant and *vice versa* (Widders *et al.*, 1989a; Corbeil, 1990) indicating the importance of these receptor proteins for resistance to serum killing. In the presence of immunoglobulin, high binding isolates were also more resistant to phagocytosis by bovine polymorphonuclear leukocytes than were low binding isolates.

1.1.2.6 Gene Cloning of *H. somnus*

Corbeil *et al.* (1988) constructed a genomic library of a virulent strain of *H. somnus* in the cosmid vector pHC79. Five clones were found to express proteins which co-migrated with *H. somnus* surface antigens. Three clones expressed both a 120 kDa antigen and a 76 kDa antigen, one clone expressed only the 76 kDa antigen and the other clone expressed a 60 kDa antigen. Both the *H. somnus* 120 kDa antigen and the recombinant 120 kDa antigen have shown immunoglobulin Fc binding activity. Restriction endonuclease mapping demonstrated that the genomic inserts of clones expressing the 76 kDa antigen shared a common 28.4 kb region, the three clones expressing 120 kDa antigen shared an

additional 7.0 kb region and the clone which expressed the 60 kDa antigen was similar to the maps of the other four plasmids. They suggested that these recombinants which express these proteins should be useful for study of protective immunity in bovine haemophilosis, as these antigens reacted with protective convalescent-phase serum. Cole *et al.* (1992) showed that the 270, 120 and 76 kDa antigens were not present in four serum-sensitive isolates from asymptomatic carriers but were present in two serum-resistant virulent strains tested by Western blotting. Southern blotting results indicated that a 13.4 kb region of DNA was missing from the four serum-sensitive strains but not from the two serum-resistant strains. The 13.4 kb segment contained an open reading frame of at least 4.5 kb which contained two 1.5 kb tandem direct repeats. The coding sequence for the 76 kDa protein starts in one of tandem direct repeat and continues beyond the other tandem direct repeat which has flanking inverted repeats similar to insertion elements. The duplicated tandem direct repeats are thus essential to the expression of a hydrophilic 76 kDa surface protein which is associated with serum resistance and virulence (Cole *et al.*, 1993).

The gene encoding a 15 kDa peptide of *H. somnus* which reacts strongly with antibodies against *H. somnus* has been investigated by Theisen and Potter (1992). They found that this gene is the second of a transcriptional unit. The first gene codes for a protein of 17 kDa. They also showed that these two proteins are present in purified ribosomes from *H. somnus*. These 15 kDa and 17 kDa polypeptides show 89% similarity to *E. coli* ribosomal protein S9 and 94% similarity to the *E. coli* ribosomal protein L13 respectively. Functional homology between the 15 kDa protein of *H. somnus* and the S9 protein of *E. coli* was also demonstrated.

Theisen *et al.* (1992) have investigated the 40 kDa lipoprotein, LppA. The gene encoding this lipoprotein has been cloned and the nucleotide sequence determined. They found that this recombinant lipoprotein product was similar to other bacterial lipoproteins. In an another study, Theisen *et al.* (1993) investigated an antigenic 40 kDa Congo red-binding lipoprotein named LppB. The aromatic dye Congo red has been used to demonstrate the virulence activity of pathogenic bacteria as the ability to bind Congo red has a strong correlation with virulence (Prpic *et al.*, 1983). LppB is predominantly present in the outer membrane fraction of *H. somnus* and they suggested that this 40 kDa surface antigen could be a vaccine candidate.

Won and Griffith (1993) constructed a genomic library of *H. somnus* in plasmid pUC19 and 45 recombinants expressed proteins which were recognised by bovine antiserum in western blots. Ten of the recombinants expressing a 31 kDa protein lysed bovine erythrocytes. They suggested this 31 kDa protein as a haemolysin but the findings were not conclusive.

1.1.2.7 Diagnosis and strain differentiation

When the first isolation of *H. somnus* was reported by Kennedy *et al.* (1960), the organism was identifed by cultural and biochemical properties. Subsequently several investigators have isolated and identified *H. somnus* by similar cultural, biochemical and fermentation properties but they have reported a varicty of results as tabulated in **Tables 1.1** and **1.2**. The tables also show that *H. somnus* is biochemically relatively inactive.

The cellular, cultural and nutritional properties of *H. somnus* have been described earlier (**sections 1.1.1.2 - 4**). The isolation of *H. somnus* is consistently hampered by overgrowth of contaminants such as *Actinomyces pyogenes* which are present in the specimens (Miller and Barnum, 1983). Paradoxically, Corbeil *et al.* (1985b) have reported that *Actinomyces pyogenes* and some other bacteria in the normal bovine flora enhance the growth of *H. somnus*. To overcome this problem, Slee and Stephens (1985), Brewer *et al.* (1985) and Brewer *et al.* (1986) developed various selective media containing antibiotics to preferentially enhance growth of *H. somnus* (see **section 1.1.7**).

Conventionally, identification of *H. somnus* is based on cultural, colonial and cellular characteristics (Humphrey and Stephens, 1983). Stephens *et al.* (1983) have compared *H. somnus* and related bacteria morphologically, biochemically, antigenically and cytochemically, and found *H. somnus*, *Haemophilus agni* and *Histophilus ovis* to be related to each other (**Table 1.1**).

Groom *et al.* (1986) evaluated the ability of the API ZYM system, a commercially available semiquantitative micromethod which detects 19 preformed enzymes, to identify *H. somnus* isolates. Although the method did not differentiate *H. somnus* from the above related bacteria they suggested that this system is more rapid than conventional biochemical methods as an identification method. The test did not differentiate isolates of *H. somnus* from different anatomical locations of cattle (Groom *et al.*, 1986). Cousins and Lloyd (1988) have confirmed the report of Groom *et al.* (1986) that the API ZYM system is a readily available, rapid and efficient method not only to identify *H. somnus* but together with a few conventional tests e.g. catalase, is also suitable for differentiation from *A. seminis*. Corbel *et al.* (1986) have used pre-formed enzymes to identify *H. somnus* which are positive for alkaline phosphatase, cytochrome oxidase, β-glucosidase, β-glucuronidase and hippurate hydrolase and the pattern differentiated the genera of *Actinobacillus, Haemophilus, Histophilus, Pasteurella*, and *Taylorella*.

The RapID NH system is designed for the rapid identification of species of *Neissseria* and *Haemophilus* isolated from humans. Salmon *et al.* (1993) evaluated its

applicability to identify *H. somnus* and related bacteria and found that the system could be used to identify *H. somnus* accurately and conveniently.

Brown *et al.* (1972) have assessed a microtiter complement fixation test (CFT) for its applicability for identification of *H. somnus*. They reported that CFT is a valuable tool for diagnostic purposes and for epidemiological studies as well as for use in selection of susceptible experimental calves and for evaluation of immune response. They also claimed that CFT is superior to tube agglutination, gel precipitation or indirect haemagglutination procedures for these studies.

The microagglutination test (MAT) is very sensitive serological method for diagnosis of reproductive and respiratory infections due to *H. somnus* (Corbeil *et al.*, 1986). However, the reliability of the test has been questioned as clinically normal cattle may develop antibodies to *H. somnus* (Humphrey and Stephens, 1983). Hoblet *et al.* (1989) showed that while experimental intrauterine inoculation of *H. somnus* into virgin heifers induced vulvovaginitis, seroconversion, as measured by MAT, was not a reliable indicator of infection. Another serological method, enzyme linked immunosorbent assay (ELISA) was developed and used to detect antibody responses to challenge with *H. somnus* by Widders *et al.* (1986) and they showed that both MAT and ELISA were not useful for detection of IgM but ELISA was useful in measurement of IgG2 to *H. somnus*.

There are two common immunodominant outer membrane proteins (OMP) of 76 kDa and 40 kDa which have been detected in *H. somnus* isolated from TEME, pneumonia, reproductive failure or asymptomatic carriers and which were recognized by immunoblotting with all convalescent sera tested. It was proposed these antigens of *H. somnus* could be important elements of a subunit vaccine or of an immunodiagnostic assay (Corbeil *et al.*, 1987).

Yarnell and Corbeil (1989) showed that the antibody response to the 270 kDa Fc receptor antigen in infected animals could be easily detected. With a protein A- peroxidase conjugate, they showed that it was possible to distinguish between normal cattle (culturally-negative for *H. somnus* or asymptomatic carriers), animals with *H. somnus* disease and animals with disease due to *P. haemolytica* or *P. multocida*. Thus this antigen may be a useful diagnostic antigen.

Corbeil *et al.* (1991) showed a characteristic OMP antigen of *H. somnus* of 39 kDa which only cross-reacted with *Haemophilus agni,* suggesting a good immunodiagnostic antigen. Tagawa *et al.* (1993a) have purified the major OMP (40 kDa) of *H. somnus*. They found considerable similarity between its amino terminal sequence and those of porin

proteins from other gram-negative bacteria. They also reported antigenic heterogeneity of *H. somnus*. The major OMP possesses at least five distinct epitopes. Three surface-exposed epitopes include a conserved epitope with potential for development as a vaccine and for a diagnostic test and two variable epitopes responsible for antigenic differences among strains. The other two epitopes are well conserved among strains but not exposed on the cell surface (Tagawa *et al.*, 1993b). A major 37 kDa heat-modifiable OMP which elicits an antibody response in *H. somnus*-infected animals is a common antigen among strains and is structurally related to the OmpA protein of *E. coli* (Tagawa *et al.*, 1993c). A 17.5 kDa OMP of *H. somnus* was shown to contain a cell surface-exposed epitope which is specific for *H. somnus*, *H. agni* and *H. ovis* and antibody to the 17.5 kDa OMP was present in convalescent-phase sera from calves with experimental pneumonia (Tagawa *et al.*, 1993d). The reactivity of monoclonal antibody (MAb 27-1) with 37 kDa OMP of *H. somnus* showd five different patterns suggesting that is a efficient subtyping system (Tagawa *et al.*, 1993c).

The recent developments in DNA technology have also been applied to identification and differentiation of *H. somnus* strains. The random amplified polymorphic DNA (RAPD) PCR assay revealed genetic polymorphisms among eight virulent and eight avirulent strains of *H. somnus* (Myers *et al.*, 1993). The RAPD markers were common to all *H. somnus* strains whereas other bacterial species tested (*H. ovis*, *H. agni*, *A. seminis*, *P. haemolytica* and *E. coli*) shared no RAPD markers with *H. somnus*. According to Eaglesome *et al.* (1992) and Myers *et al.* (1993) these markers hold promise as diagnostic probes for rapid detection of *H. somnus*. Fussing and Wegener (1993) have compared biotyping and different molecular typing methods for *H. somnus*. They found 21 different biotypes, 12 different plasmid profiles, 33 different restriction endonuclease patterns with *Taq*1 restriction enzyme and 16 different ribotype patterns after *Eco*R1 restriction. In their study, isolates from different animals in the same herd and isolates from different anatomical sites in same animal gave similar results for REA typing, ribotyping and plasmid profiles but different biotyping profiles.

1.1.2.8 Prevention and Control

Treatment. Oxytetracycline was commonly used for treatment and when administered intravenously has been effective in treating the septicaemic form of *H. somnus* (Eaglesome *et al.*, 1992). Harris and Janzen (1989) observed that reproductive diseases due to *H. somnus* were responsive to antibiotic treatment unless there was involvement of other complicating agents. Eaglesome *et al.* (1992), citing Luginbuhl and Kupfer (1981), reported that intrauterine antimicrobial therapy had little effect on *H.*

somnus in cervical mucus in the post partum period. Based on sensitivity results, Klavano (1980) used penicillin and streptomycin to improve fertility of a cattle herd where *H. somnus* was frequently isolated from cervicovaginal mucus. Donkersgoed *et al.* (1990) reported that prophylactic mass medication with antibiotics in the feed or giving injections of long acting antimicrobials during the periods of greatest risk of *H. somnus* infection may reduce losses from Haemophilosis. *H. somnus* has been isolated from bovine semen or seminal fluid (Janzen *et al.*, 1981) and although the pathogenicity of the semen isolate was not proven, this might be a source of infection for cows. To overcome this danger, Shin *et al.* (1988) used two combinations of antibiotics (gentamycin (500 μg/ml), tylosin (100 μg/ml) and linco-spectin (300/600 μg/ml) or penicillin (500 iu/ml), dihydrostreptomycin (2000 μg/ml) and polymyxin-B sulphate (1000 iu/ml) to control *H. somnus* in artificially-infected semen.

Vaccination. Although administration of two doses of bacterin reduces morbidity and overall mortality, the efficacy of its protection against TEME was incomplete (Humphrey and Stephens, 1983; Eaglesome *et al.*, 1992). Donkersgoed *et al.* (1990) showed that peracute *Haemophilus* septicaemia and TEME were observed a few weeks after arrival in the feedlot which suggest that routine single immunisation with a *H. somnus* bacterin and mass treatment of calves with oxytetracycline on arrival at the feedlot did not eliminate carriers of *H. somnus*.

Immunisation with killed whole *H. somnus* has had very little effect on the naturally infected vaginal carriers of cattle (Widders *et al.*, 1989b). Primal *et al.* (1990) vaccinated cattle with an outer membrane anionic antigen fraction isolated from *H. somnus* and obtained some protection against experimental pneumonia. Gogolewski *et al.* (1988) have reported that monospecific bovine polyclonal antibody to 40 kDa OMP from *H. somnus* gave passive immunity to calves against experimental pneumonia. Corbeil *et al.* (1991) observed that the antibody to 40 kDa OMP cross-reacted strongly with species within the family *Pasteurellacae* and it was suggested that the purified antigen could be a useful component in a vaccine against the major aetiological agents of the bovine respiratory disease complex, namely *P. haemolytica, P. multocida* and *H. somnus* (Corbeil *et al.*, 1991). Widders *et al.* (1986) studied the immune response against *H. somnus* experimental abortion. They reported that there was a 10-fold increment and persistent response for IgG2 after challenge. They pointed out that IgG2 antibody may have a role in limiting haematogenous dissemination of *H. somnus*.

1.2 *HISTOPHILUS OVIS*

1.2.1 *Histophilus ovis* - the organism

The name *Histophilus ovis* has been applied to Gram-negative pleomorphic coccobacilli isolated from sheep with a variety of disease conditions including septicaemia, pyaemia, polyarthritis, mastitis, metritis, abortion, epididymitis and orchitis and this organism shows similar properties to that of *H. somnus* (Humphrey and Stephens, 1983). Studies on *H. ovis,* however, have been very limited.

1.2.1.1 History and Nomenclature

Roberts (1956) first isolated the organism from a case of ovine mastitis and he suggested the name *Histophilus ovis* as it was 'fond of tissues' and was isolated from a sheep. He placed it in this new genus of the tribe *Haemophileae* as the properties of this organism showed close similarities to those of this tribe (**Table 1.1**). Stephens *et al.* (1983) included this organism in the *Haemophilus-Histophilus* group on the basis of their similar morphological, biochemical, antigenic and cytochemical properties with *H. somnus* and *H. agni* but *H. ovis* has been omitted from the **Approved Lists of Bacterial Names** on the grounds of non-availability of representative cultures (Kilian and Biberstein, 1984). Consistently, subsequent DNA-DNA hybridisation studies have shown that *H. somnus*, *H. ovis* and *H. agni* are genetically homogenous species (Walker *et al.*, 1985; Piechulla *et al.*, 1986). Now a conflict has arisen as some groups e.g. Lees *et al.* (1994) have described isolates from both bovine and ovine origins as *Haemophilus somnus*.

1.2.1.2 Bacteriological characteristics

H. ovis is a Gram-negative rod which shows different morphological forms with the age of the culture. It is non-motile, non-capsulated and non-spore forming. The cells from young liquid cultures of *H. ovis* were rods with rounded ends about 0.4 or 0.5 µm wide and from 1-3 µm long (Roberts, 1956). Similar observations were made by Webb (1983a) and he did not detect flagella. Most properties of *H. ovis* are similar to those of *H. somnus* (**Table 1.1**).

1.2.1.3 Cultural characteristics

Colonial morphology. The colonies on blood containing medium are pinpoint round, glistening and transparent after incubation at 37 °C for 24 h in reduced oxygen tension. When incubated for 48 h, the colonies become 1-2 mm in diameter (Stephens *et al.*, 1983; Webb, 1983a).

Pigmentation. The yellow colour of colonies was apparent when smeared on white paper but was not visible on the agar (Roberts, 1956). Stephens *et al.*(1983) have shown yellow pigmentation of cell lysates but Webb (1983a) was unable to show pigment production.

Haemolytic activity. There was no haemolysis of ovine blood (Roberts, 1956; Webb, 1983a; Ward *et al.*, 1995) or bovine blood (Ward *et al.*, 1995) by *H. ovis* isolates.

1.2.1.4 Growth requirements

Nutritional requirements. The first isolation of *H. ovis* was made on blood agar and cooked meat medium (Roberts, 1956) but the source of blood was not mentioned. Webb (1983a) used nutrient agar with 10% (v/v) sheep blood and peptone or peptone water with 10% sterile sheep serum or meat particles. Stephens *et al.* (1983) used blood agar supplemented with 0.5% (w/v) yeast extract and 7% (v/v) bovine blood for comparative studies of *H. ovis*. There was no difference in growth on Brain Heart Infusion agar with added 5% (v/v) blood when either ovine or bovine blood was used (Ward *et al.*, 1995).

CO_2 requirement. Roberts (1956) used 10% (v/v) CO_2 for the first isolation of *H. ovis* and reported that growth would not occur in the absence of oxygen. Webb (1983a) used a candle jar for *H. ovis* growth and Stephens *et al.* (1983) showed that 10% (v/v) CO_2-90% (v/v) air was the best atmosphere for growth. Ward *et al.* (1995) assessed haemolytic activity by incubating *H. ovis* isolates in a candle jar.

Temperature. Roberts (1956) isolated *H. ovis* by incubating at 37 °C. Webb (1983a) obtained optimal growth at 37 °C and was unable to show visible growth at 22 °C and 45 °C.

1.2.1.5 Biochemical characteristics

Roberts (1956) reported that *H. ovis* isolates were positive for indole production, nitrate reduction and negative for catalase, H_2S production, gelatine liquefaction and did not change the colour of litmus milk. They produced acid but no gas from xylose, glucose, laevulose, mannose, mannitol and sorbitol and there was no reaction with arabinose, rhamnose, galactose, sucrose, maltose, lactose, trehalose, raffinose, inulin, dextrin, dulcitol, salicin or inositol. Webb (1983a) compared the biochemical properties of 17 *H. ovis* strains isolated from sheep with different disease entities and he obtained 100% positive results for indole, ornithine decarboxylase and the O/F test was fermentative. The nitrate test was positive for 94% of isolates but other tests, catalase, oxidase, nitrite, MR, VP, H_2S, gelatine, urease, arginine dihydrolase, lysine decarboxylase, litmus milk, citrate and KCN were found to be negative. For fermentation reactions, all 17 isolates produced acid from glucose, fructose, galactose and mannose and were negative for arabinose, sucrose, lactose, trehalose, inulin, glycerol, adonitol, dulcitol, inositol and raffinose. The reactions were variable for xylose, mannitol, sorbitol, salicin, rhamnose and maltose. Stephens *et al.* (1983) and Ward *et al.* (1995) have also shown the variability of biochemical reactions.

1.2.1.6 Storage of *H. ovis* isolates

Liquid paraffin-overlaid blood agar stab cultures were used for storage of *H. ovis* by Roberts (1956). The yolk sacs of 7 day old embryonated eggs were inoculated with *H. ovis* and incubated for 24 h and harvested yolk sac in 1 ml portions were stored at -70 °C (Stephens *et al.*, 1983). Ward *et al.* (1995) used lyophilisation or a mixture of phosphate buffered saline (pH 7.2) 40% and glycerol 60% and storage at -70 °C as a method of preservation of cultures.

1.2.1.7 Antimicrobial sensitivity

H. ovis has been reported as sensitive to penicillin, aureomycin, terramycin, tetracycline and chloromycetin, moderately sensitive to streptomycin and resistant to sulphamezathine (Roberts, 1956) and sensitive to ampicillin, chloramphenicol, erythromycin, furacin, gentamycin, neomycin, polymyxin B, tetracyclines, triple sulpha and trimethoprim-sulphamethoxazole (Beauregard and Higgins, 1983).

1.2.1.8 Plasmids

In one report, all *H. ovis* isolates examined (n=15) of ovine origin showed the presence of plasmids (Kirkham *et al.*, 1989). These isolates contained from 1-6 plasmids ranging in size from 3.9 - 90 kb but no single plasmid was present in all *H. ovis* isolates. No properties were attributed to the plasmids. Plasmid profile analysis has successfully been applied to strain differentiation of *H. ovis* by Kirkham *et al.* (1989) and interestingly, all *H. ovis* (n=15) and similar isolates from ovine origin (*H. agni*) (n=9) contained plasmids but none of the similar isolates of bovine origin (n=20) showed plasmids.

1.2.1.9 Taxonomic status of *H. ovis*

The taxonomic status of *H. ovis* is unclear but the organism resembles *Haemophilus somnus*, an organism causing similar disease conditions in cattle, in its cultural, biochemical and antigenic properties. Stephens *et al.* (1983) suggested that these bacteria and an organism referred to as *Haemophilus agni* should be considered as a single *Haemophilus-Histophilus* group and they pointed out the problem of differentiation of strains within the group. Confusingly, there has been a trend to identify both bovine and ovine isolates of these organisms in North America as *Haemophilus somnus* (Ward *et al.*, 1995) and in Australia as *Histophilus ovis* (Stephens *et al.*, 1983; McGillivery *et al.*, 1986). DNA-DNA hybridisation studies have shown that *H. somnus* and *H. ovis* are genetically homogeneous (Piechulla *et al.*, 1986) but others have suggested that, on the basis of restriction enzyme analysis, biotyping and outer membrane protein profiles (Walker *et al.*, 1985; Ward *et al.*, 1995), bovine and ovine isolates should be considered as separate groups.

1.2.1.10 Serological cross-reactivity with other bacteria

Stephens *et al.* (1983) showed that there were two common antigens shared by *H. ovis* and *H. somnus* and *H. agni* in gel immunodiffusion tests. Similarly, it has been reported that there were no differences in degree of agglutination in slide agglutination tests between *H. ovis* and *Actinobacillus seminis* with sera against either organisms (Webb, 1983a).

1.2.2 Disease caused by *Histophilus ovis*

1.2.2.1 Epidemiology

H. ovis was first reported from a case of mastitis of ewes in Australia. A similar organism, *Haemophilus agni*, was isolated by Kennedy *et al.* (1958) in America from septicaemic disease of lambs. *H. ovis* has been isolated from cases of polyarthritis, epididymo-orchitis, meningoencephalitis, pneumonia, septicaemia, mastitis and metritis in Australia (Philbey *et al.*, 1991) and from vaginal discharge in ewes (Higgins *et al.*, 1981) and from ovine mastitis (Beauregard and Higgins, 1983) in Canada. It was first isolated in the UK by Low and Graham (1985) from a ram with epididymitis and orchitis and subsequently from a case of ovine abortion (McDowell *et al.*, 1994) and cases of thrombotic meningoencephalitis in lambs (Cassidy *et al.*, 1997). In a study by Lees *et al.* (1990) in Canada, 9.1% of 50 days old lambs were positive for *H. ovis*. They also showed that the presence of cattle on the farm was a predisposing factor for *H. ovis* in the flock.

1.2.2.2 *H. ovis* - associated diseases

The disease syndromes caused by *H. ovis* in sheep are very similar to those of haemophilosis in cattle. The disease syndromes are mastitis (Roberts, 1956; Beauregard and Higgins, 1983), meningoencephalitis (Philbey *et al.*, 1991; Cassidy *et al.*, 1997), septicaemia (Kearney and Orr, 1993), epididymitis (Low and Graham, 1985) abortion (McDowell *et al.*, 1994) and vaginal discharges (Higgins *et al.*, 1981).

The first reported isolation of *H. ovis* was made from a case of ovine mastitis (Roberts, 1956). The ewe was dead and was at the terminal part of gestation. The left half of the udder was in the normal lactating state but the right half was very enlarged, turgid and contained brownish purulent fluid. The skin was tightly stretched and had a patchy blue colour. A report of mastitis in Canada due to *H. ovis* described an outbreak in a flock (Beauregard and Higgins, 1983).

Sudden death is the main clinical feature of the encephalitis form of the disease. The other clinical signs are recumbency, opisthotonus, hypersalivation and congestion of the conjunctival mucosae. The neurological signs like nystagmus and weak paddling when stimulated are observed at the terminal stages. The gross lesions are found not only in the brain but also in kidney, liver and heart. The main microscopic features are vasculitis and thrombosis especially in small blood vessels (Philbey *et al.*, 1991; Cassidy *et al.*, 1997).

The septicaemic form of *H. ovis* was characterised by an outbreak showing dullness disinclination to move, lameness and death within 24 h. The necropsies showed multiple haemorrhages throughout the carcass especially in the skeletal muscle and on serosal surfaces. Congestion of subcutis, pale liver and kidney, and excess greenish fluid in joints were the other gross lesions. The histological features were focal hepatic necrosis and microabscesses in liver, skeletal muscle and heart (Kearney and Orr, 1993).

The reports of epididymitis due to *H. ovis* were of chronic cases. Early changes included the production of yellow mucoid semen with <1% live sperm and the presence of abnormal appearances of sperm. Later, the scrotum was extremely swollen, red and painful to touch and the animals showed elevated rectal temperature. *H. ovis* has been isolated from seminal excretions and epididymal tissues (Low and Graham, 1985). The average daily gain was not affected by *H. ovis* status but was influenced by breed of ram. There was no relationship between *H. ovis* status and lambing percent or the percent of abortions and stillbirths but there was significant association with the percent of ewes which failed to lamb suggesting that *H. ovis* may influence ewe fertility earlier rather than later in gestation (Lees *et al.*, 1990).

1.2.2.3 Experimental infection

Roberts (1956) found that there were no harmful effects for mice, guinea pigs and rabbits after injection of their *H. ovis* isolate by subcutaneous and intraperitoneal routes but he successfully established the organism in the ovine udder and reproduced the clinical signs of mastitis. Webb (1983b) reproduced the clinical diseases i.e. epididymitis, polyarthritis, mastitis and abortion in sheep with *H. ovis* isolates obtained from their respective field cases. Studies on experimental infection of *H. ovis* are limited and there are no reports on site specificity of *H. ovis* isolates.

1.2.2.4 Diagnosis

The diagnosis of diseases due to *H. ovis* has been made by the isolation of the causative agent (Roberts, 1956; Beauregard and Higgins, 1983; Kearney and Orr, 1993; Cassidy *et al.*, 1997). The identification of *H. ovis* has been confirmed by cultural and biochemical properties (**Table 1.1**) but the properties are very similar to those of *H. somnus* and isolates originating from sheep were called *H. ovis*. The ELISA technique for the detection of antibodies to *H. ovis* has been applied with heat extracted antigen to evaluate the association of epididymal lesions in rams with a serological response to *H.*

ovis (Walker *et al.*, 1988). Cassidy *et al.* (1997) used avidin-biotin-peroxidase technique to detect *H. somnus* antigen in lamb tissue sections with antiserum to the Fc receptor of *H. somnus* as primary antibody. This finding also shows the close serological relationship between *H. ovis* and *H. somnus*. None of these tests were able to distinguish *H. ovis* from *H. somnus*.

1.3 *ACTINOBACILLUS SEMINIS*

1.3.1 *Actinobacillus seminis* - the organism

Actinobacillus seminis is also a Gram-negative pleomorphic bacterium that shows similar phenotypic properties to *H. somnus* (Humphrey and Stephens, 1983). This organism was first reported from ovine epididymitis in Australia (Baynes and Simmons, 1960) and is now recognised as an important cause of epididymitis and infertility in rams.

1.3.1.1 History, nomenclature and taxonomic status

The first reported isolation of *A. seminis* was made in Australia by Baynes and Simmons (1960). They isolated this organism from semen of rams which had epididymitis and found that its characteristics were similar to those of the genus *Actinobacillus*. Subsequently *A. seminis* has been associated with various reproductive problems of rams (Humphrey and Stephens, 1983). As the taxonomic placement of this organism was not clear, Phillips (1984) suggested exclusion of this organism from the genus *Actinobacillus* without providing a taxonomic placement. In 1990, Sneath and Stevens (1990) proposed that *A. seminis* should be regarded as a new species in the genus of *Actinobacillus* on the evidence from numerical taxonomic analysis and DNA-DNA hybridisation results.

1.3.1.2 Bacteriological characteristics

The bacteriological properties of *A. seminis* are well documented (Baynes and Simmons, 1960; van Tonder, 1979; Hajtos *et al.*, 1987; Sneath and Stevens, 1990). This organism is a pleomorphic Gram-negative bacillus that is approx. 1-4 μm long and 1 μm in width and filamentous forms 10-12 μm long have been described. It is non-acid-fast, but occasionally retains fuchsin in semen smears especially when intracellular. *A. seminis* is non-motile (Baynes and Simmons, 1960; van Tonder, 1979), non-capsulated and non-spore-forming (Hajtos *et al.*, 1987).

1.3.1.3 Cultural characteristics

Colonial morphology. The colonies on bovine blood agar with 0.5% yeast extract are pin-point after 24 h incubation at 37 °C in 10% CO_2. After 48 h, the colonies become greyish-white, shiny, circular, non-haemolytic low and convex, 1-2 mm diameter with an entire edge. After 72 h, the colonies are flattened and umbonate greyish-white centre and transparent peripheral zone (Hajtos *et al.*, 1987). By colony appearance, *A. seminis* cannot readily be differentiated from *H. somnus* and *H. ovis*.

Haemolytic activity. No haemolysis was observed on bovine blood agar (van Tonder, 1979; Hajtos *et al.*, 1987).

1.3.1.4 Growth requirements

Nutritional requirements. Growth depends on the presence of serum or blood and, without these, growth is poor. Ovine blood (Baynes and Simmons, 1960), equine blood (van Tonder, 1979) and bovine blood (Hajtos *et al.*, 1987) have been used. Growth is poor on ordinary media and no growth occurs on MacConkey agar (Baynes and Simmons, 1960; van Tonder, 1979; Hajtos *et al.*, 1987).

CO_2. Although growth occurs acrobically and anaerobically, it is more luxuriant in the presence of 10% (v/v) CO_2 (Baynes and Simmons, 1960; Hajtos *et al.*, 1987). van Tonder (1979) has obtained better growth after incubating in an atmosphere of 20% (v/v) CO_2 and 80% (v/v) air.

Temperature. Baynes and Simmons (1960) isolated *A. seminis* after incubating at 37 °C and in, subsequent reports, the same incubation temperature has been used (van Tonder, 1979; Hajtos *et al.*, 1987).

1.3.1.5 Biochemical properties

Although, Baynes and Simmons (1960) and van Tonder (1979) have reported *A. seminis* as a biochemically inactive bacterium, Hajtos *et al.*(1987) found that it is biochemically active in suitable media. Positive reactions are given for catalase, oxidase, nitrate, hydrogen sulphide production and negative for indole, urease, phosphatase and β-galactosidase. Strains are also 100% fermentative for only arabinose, glucose and xylose (Hajtos *et al.*, 1987).

1.3.1.6 Antimicrobial sensitivity

Isolates obtained by Baynes and Simmons (1960) were sensitive to penicillin, streptomycin, chloramphenicol, aureomycin, terramycin, erythromycin, tetracycline and novobiocin and resistance and partial resistance were observed for bacitracin and neomycin respectively. According to Hajtos *et al.*(1987), their isolates were sensitive to penicillin, chloramphenicol, oxy- and chlortetracycline, polymyxin and nitrofurantoin, some strains were sensitive to erythromycin, streptomycin and neomycin and all the strains were resistant to bacitracin and vancomycin.

1.3.1.7 Serological cross reactions

Rahaley (1978) showed the cross reaction of *A. seminis* with *H. ovis* and *Brucella ovis* by a cross-absorption complement-fixation test. *A. seminis* antigens produced two lines of partial identity with antiserum against *H. somnus* in gel immunodiffusion tests (Stephens *et al.*, 1983).

1.3.2 Disease caused by *Actinobacillus seminis*

1.3.2.1 Epidemiology

The main clinical diseases caused by *A. seminis* are epididymitis and orchitis and these occur only in rams. The first isolation of *A. seminis* was from cases of epididymitis in rams in Australia (Baynes and Simmons, 1960) and most subsequent isolations were made from rams with reproductive disorders in the USA (Livingston and Hardy, 1964), in South Africa (van Tonder and Bolton, 1968) and in Europe (Hajtos *et al.*, 1987). It was first isolated in the United Kingdom in 1991 (Heath *et al.*, 1991). A recent survey in the UK showed it to be present in the semen of 19% of infertile rams and in 4.5% of samples overall (Low *et al.*, 1995). *A. seminis* has also been isolated from polyarthritis and posthitis in lambs in Australia (Watt *et al.*, 1970), and from cases of abortion in sheep and goats in South Africa (van Tonder, 1973). Although the common natural host of *A. seminis* is sheep, there are reports of its isolation from cattle (Dixon *et al.*, 1983).

1.3.2.2 *A. seminis*-associated epididymitis

The clinical signs of depression, loss of appetite and swollen testes were observed by Baynes and Simmons (1960). The disease appears as chronic and acute forms of orchitis or epididymo-orchitis or epididymitis. The acute form is characterised by intense swelling, pain and heat of scrotal contents, accompanied by a severe systemic reaction. In some cases the scrotum may rupture and discharges greyish white to yellow pus (van Tonder, 1973). The semen of affected cases contains pus which yields heavy cultures of *A. seminis* (Heath *et al.*, 1991).

1.3.2.3 Experimental infections

A. seminis had no effect on mice and guinea pigs after experimental inoculations by the intraperitoneal and intramuscular routes but it became successfully established and reproduced epididymitis on experimental inoculation into the epididymis and testis of rams (Baynes and Simmons, 1960).

1.3.2.4 Diagnosis

The first isolation of *A. seminis* was determined by cultural and biochemical properties and subsequent cases were diagnosed by complement fixation test (CFT) (Baynes and Simmons, 1960). van Tonder (1973) used CFT for the diagnosis of *A. seminis* infections saying it was more reliable than clinical, semen smear and bacteriological examination. Later, the identification of *A. seminis* was carried out using more standardised biochemical methods such as API 20 E (Erasmus, 1983; Heath *et al.*, 1991) and API ZYM (Cousins and Lloyd, 1988; Low *et al.*, 1995).

1.4 MOLECULAR APPROACHES TO MICROBIAL IDENTIFICATION AND TYPING

1.4.1 History

Since bacteria were first isolated, workers have tried to classify them according to their properties such as staining reactions, morphology, motility, nutritional requirements, acid production, fermentation reactions, pigmentation and spore formation. All these are phenotypic characteristics and may be highly variable often due to differences in the way the organisms are grown. The major drawback of this sort of system is that the tests derived for one group of organisms are not always useful for other groups. An ideal system should be able to identify and type the vast majority of strains encountered, have good discrimination with the ability to recognise a reasonable number of types, show good reproducibility over a long period of time and in different centres, be readily applicable not only to laboratory collections of strains but also to natural isolates and should not be too intricate, time consuming or expensive. There are several conventional methods for microbial typing.

1.4.1.1 Biotyping

In this method, bacterial species and strains are differentiated by cultural and biochemical characteristics. It has been the initial step of identification almost all bacterial species including *H. somnus*, *H. ovis* and *A. seminis* as mentioned in **section 1.1**. These characteristics may include colonial morphology, growth requirements, fermentation ability, carbon source utilisation and antibiotic resistance. Colony morphology may be highly variable and biochemical reactions more dependable but these may difficult to interpret. Fermentation reactions depend on the production relevant enzymes by the bacterial isolate. The standardised method of biochemical reactions are commercially available as API 20 E (BioMerieux, Marcy l'Etoile, France) which has been used to identify *A. seminis* (Erasmus, 1983). Recently, a computer based automated system for detection and typing of bacteria with 95 different carbon-based substrates has been introduced by Bio-Log (Bio-Log Inc., Hayword, Ca., USA). The API ZYM system (BioMerieux) has been developed to detect bacterial enzymes with a view to identification and typing of bacteria (Humble *et al.*, 1977). This method has been used for identification and differentiation of *H. somnus*, *H. ovis* and *A. seminis* (Groom *et al.*, 1986; Cousins and Lloyd, 1988). Antibiograms are easy to perform but may be variable due to gain or loss of resistance (R) plasmids.

1.4.1.2 Phage typing

Phage typing is a method for bacterial strain identification and differentiation that is based on sensitivity to defined collections of bacteriophages which have been selected to provide the maximum sensitivity for differentiating strains within a particular species. Phage typing schemes are highly sensitive and have been developed for numerous bacterial genera, many of which have not yet been typed successfully by other means. The method is technically demanding, however, and cannot be applied to a new organism.

1.4.1.3 Bacteriocin typing

Bacteriocins are bactericidal substances, normally proteins, which are active against different strains of bacteria. Bacteriocin typing is performed by testing the sensitivity of unknown strains to bacteriocins produced by a set of standard strains and *vice versa*. The production of bacteriocins or sensitivity to them are relatively stable and these bacteriocin may be encoded by transmissible R plasmids. The involvement of extensive labour and the requirement of considerable development work before application to a new species are disadvantages faced by this method.

1.4.2 Molecular techniques

The fundamental macromolecules like nucleic acids, proteins, and lipopolysaccharides carry important information about the particular organism. The sequence and organisation of these macromolecules should provide a universal molecular approach to microbial identification and typing of any organism. Recently developed techniques used to isolate and characterise macromolecules in molecular biology have become increasingly rapid and simple, to the extent that they are now readily available for any laboratory. Although the examination of protein and lipopolysaccharide profiles has been used to type a variety of different bacteria, there is a limitation to these techniques in that they analyse the phenotype rather than the genotype of a particular organism. The analysis of the genotype of an organism does not rely on the expression of particular genes encoding proteins or lipopolysaccharides, and it is not dependent on phenotypic variation. The genotypic characterisation of bacteria is superior to phenotypic characterisation (Kerr, 1994).

1.4.2.1 Protein analysis

Proteins plays a role of structural and metabolic function in any organism. The diversity of these proteins among organisms provides potential identification and typing systems. The electrophoretic patterns of these proteins are usually highly complex and the profiles generated may vary according to the method of sample preparation. Several techniques have been applied to detect protein profiles.

1.4.2.2 Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MEE) is used to differentiate soluble proteins, especially metabolic enzymes present in organisms. This technique was developed originally for analysis of genetic diversity of mammalian populations and now is used widely for the discrimination of closely-related microorganisms (Towner and Cockayne, 1993). The expression of enzymes is a phenotypic character, therefore all the steps involved in sample preparation should be standardised. MEE is a useful technique for epidemiological investigations. This method has been applied for differentiation not only of bacteria but fungi and protozoa (Towner and Cockayne, 1993).

1.4.2.3 Polyacrylamide gel electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis (PAGE) is applied to detect protein, polypeptide or peptide profiles of microorganisms. PAGE can be used with or without sodium dodecyl sulphate (SDS-PAGE), in which proteins are resolved under denatured or non-denatured conditions respectively. SDS-PAGE is superior to PAGE due to better resolution and reproducibility and it avoids the effect of shape and native charge of proteins on electrophoretic mobility. SDS-PAGE can be used to detect not only protein profiles but also lipopolysaccharide and lipooligosaccharide profiles. Whole-cell SDS-PAGE protein profiles have been used successfully for identification and typing of a wide range of bacterial strains. Stephens *et al.* (1983) have used SDS-PAGE of cell envelope proteins of *H. somnus* and other related bacteria to differentiate them. It has also been used for the characterisation of major outer membrane proteins of *H. somnus* (Tagawa *et al.*, 1993a). Sometimes, however, SDS-PAGE yields complex profiles not suitable for identification purposes and sometimes it produces identical profiles or reveals only minor differences in patterns between closely related strains. Another limitation of this technique is that proteins or polypeptides with similar molecular mass may co-migrate, resulting in a single band on a stained gel.

Western blotting may be performed to immobilise the resolved products of SDS-PAGE. Proteins or lipopolysaccharides in the SDS-PAGE gel are electrophoretically transferred to nylon or nitrocellulose membranes and visualised by staining. When these Western blotted membranes are treated with antiserum (immunoblotting) the antigenicity of appropriate proteins can be detected. Immunoblotting has been applied to *H. somnus* to detect and characterise the 40 kDa OMP (Corbeil *et al.*, 1991). SDS-PAGE and immunoblotting have been used to demonstrate the phenotypic phase variation of lipooligosaccharide of *H. somnus* (Inzana *et al.*, 1992). Distortion of profiles, poor resolution and transfer, high background level due to inadequate blocking and smearing of bands are problems associated with immunoblotting.

1.4.2.4 Serotyping

Serotyping is one of the oldest typing systems, and is useful for routine identification and typing of many bacterial species. Serotyping is based on reactions with specific monoclonal and polyclonal antibodies (e.g. anti-somatic, anti-flagella or anti-capsular), raised against the antigenic structures of microbes. The advantage of serotyping is that it can be applied to many different genera although the specific antisera can be applied only to a single species. It has been reported that the serotype of a microbe is a relatively stable and reliable typing marker. Problems in antiserum production and standardisation of methodology are the main disadvantages. There are several techniques of serotyping. Agglutination (slide and tube) and immunofluorescence are older methods and these suffer several problems such as non-specific reactions and the fact that interpretation of results may be highly subjective. Agglutination tests are performed to identify and serotype many Gram-negative bacteria including *Salmonella* spp. (Kaufmann, 1972) and *E. coli* (Orskov and Orskov, 1984). The slide agglutination test has been used to differentiate *H. somnus* from other related bacteria (Stephens *et al.*, 1983). Immunoblotting is a relatively new serological test that is performed in conjunction with SDS-PAGE (see above). Enzyme linked immunosorbent assay (ELISA) is a recent serological test that has been used successfully for the identification and typing of bacteria. This is a colourimetric assay which does not have most of the limitations of the above serological tests. The antigenicity of the major outer membrane protein of *H. somnus* has been analysed by ELISA using monoclonal antibody (Tagawa *et al.*, 1993a). The antigenicity of lipooligosaccharides from *H. somnus* was also detected by ELISA (Inzana *et al.*, 1988).

1.4.3 Nucleic acid analysis

1.4.3.1 Analysis of plasmid DNA

The earliest nucleic acid molecules to be examined were plasmids. There are simple and rapid methods for isolating plasmid DNA in any laboratory. The lysate of target cells is subjected to phenol and chloroform extraction for isolation of plasmid DNA which is then analysed by agarose gel electrophoresis. Easy, quick, safe and economical methods are now available commercially as kits for plasmid isolation. More detailed analysis of plasmid DNA can be performed after restriction endonuclease digestion of purified plasmid DNA. The digested product produces a fingerprint after electrophoresis in agarose gels, giving additional information and readily distinguishing unrelated plasmids of similar size. Individual DNA fragments or whole plasmids can also be compared by means of DNA-DNA hybridisation procedures (see below). Different bacterial genera have been typed by plasmid pattern analysis but the possible long-term lack of stability of plasmids in particular strains may pose a major drawback. Furthermore, plasmid analysis cannot, of course, be applied if the bacteria to be examined do not contain plasmids. For example, only 20% of Danish strains of *H. somnus* contained plasmids (Fussing and Wegener, 1993). Plasmids can also be lost or gained by strains of a particular species and so their analysis does not constitute a reliable typing procedure.

1.4.3.2 Analysis of chromosomal DNA

Restriction endonuclease analysis (REA). Chromosomal DNA is the one of most stable molecules in a living cell and analysis of the stucture of this molecule would indicate the unique properties of it. The chromosomal DNA can be treated with a restriction endonuclease enzyme and the digest resolved by standard electrophoretic methods. Such methods are called restriction endonuclease analysis (REA) which is applicable to any culturable bacterium. The drawbacks of this method are that it cannot be applied to non-culturable bacteria as a large volume of chromosomal DNA is needed, plasmid DNA can interfere with the analysis, and the complexity of the fingerprints is difficult to interpret. REA is incapable of separating DNA fragments greater than 50-100 kb in size but the method has been used for typing of *H. somnus* (Fussing and Wegener, 1993; Ward *et al.*, 1995), for the differentiation of *H. somnus* from *H. ovis* (McGillivery *et al.*, 1986; Kirkham *et al.*, 1989) and characterisation of *A. seminis* (McGillivery and Webber, 1989).

Pulsed-field gel electrophoresis (PFGE). This method was first described by Schwartz and Cantor (1984) (cited by Maslow and Mulligan, 1996), and overcomes some of the problems of REA. In this technique, rare-cutting restriction endonucleases are used and generate only a limited number of large DNA fragments. During electrophoresis, the orientation of the electrical field is changed periodically. With this method PFGE is powerful enough to resolve the large DNA fragments of 50 kb-12 Mb. The test organism is embedded in agarose plugs and the DNA is released in situ for minimising the shearing of DNA before restriction enzyme digestion. This procedure has been used for epidemiological and typing investigations with a wide range of microorganisms. The pattern of restriction fragments is characteristic for each strain and provides an estimate of the degree of genomic relationship between strains. Closely-related strains that differ by only a few bands can be identified readily by side-to-side visual comparison of the fingerprint patterns on the same gel. The discriminatory value and information generated by the technique can be increased further by the use of more than one rare-cutting enzyme, either individually or collectively. There are drawbacks to this technique too. PFGE requires specialised apparatus and experience. The extraction of high molecular mass chromosomal DNA is a difficult and time-consuming procedure especially with slow growing bacteria. In addition the technique is relatively insensitive for detecting small differences between strains. Additional bands, due to the presence of extrachromosomal plasmids or bacteriophage DNA are also possible. This technique has been successfully applied for epidemiological investigation of *Flavimonas oryzihabitans* (Liu *et al.*, 1996).

1.4.3.3 Hybridisation procedures

Nucleic acid hybridisation is done using a labelled nucleic acid probe. There are two steps involved in the hybridisation technique. First, the target nucleic acid molecule is denatured by treatment with alkali or heating and then a labelled nucleic acid probe is annealed by subsequent neutralisation or cooling. The specificity (stringency) and speed of such a hybridisation reaction can be controlled by manipulating the temperature, salt concentration, pH, probe concentration and probe size. The greater the stringency, the greater the specificity. The probe can be a specific cloned DNA fragment or a synthetic oligonucleotide. The method of labelling of the probe depends on whether the detecting system is either direct or indirect. The direct labels are radioisotopes such as ^{32}P and ^{35}S, and indirect labels include enzymes such as alkaline phosphatase and horseradish peroxidase or dyes like ethidium or fluorescein. In indirect labelling, the probe is labelled with a modifying group which subsequently binds with a reporter molecule. The radioactive labels have restrictions to use i.e. they may have short half-lives and possible health and safety hazards. The hybridisation can be applied after purification of target DNA

by agarose gel electrophoresis transferring DNA to a solid (nitrocellulose or nylon membranes) matrix by Southern blotting or in liquid phase. In general, probe assays which are based on labels that provide signal amplification (i.e. enzymes) can be expected to be more sensitive than assays based on labels that provide only a single signal per label e.g. fluorescein. The main drawback is that each individual species to be identified requires its own specific probe and this is an identification method rather than a typing system. However, it can be developed into a typing method by combining with restriction endonuclease analysis (Towner and Cockayne, 1993).

1.4.3.4 Ribotyping

The term ribotyping is used when the probe for hybridisation is based on rRNA. The rRNA gene is found to be highly conserved and so probes specific for these sequences can detect a wide range of bacteria with similar rRNA sequences. The genome of the organism is digested with a restriction enzyme (or a combination of enzymes) to give a number of bands after electrophoresis on an agarose gel. The fragments are then transferred by Southern blotting to a nitrocellulose or nylon membrane and hybridised with a labelled rRNA probe. Finally the bands are compared with different strains. There are several advantages over other typing systems. For example, the ribosomal genes are extremely stable; the commercially available rRNA from *E. coli* can be used as a universal probe when labelled. This method has been applied to type *H. somnus* isolates by Fussing and Wegener (1993) and by Ward *et al.* (1995).

The main advantages of hybridisation-based typing methods are that they are applicable to a wide range of microorganisms, commercially-available rRNA can be used as a universal probe, hybridisation patterns are reproducible and relatively simple to interpret and computer analysis can be used to compare patterns with information stored in databases. The disadvantages are that they are time consuming, have a relatively complex methodology and information is provided only about regions of the genome that hybridise with the particular probe being used.

1.4.3.5 16S rRNA sequence analysis

Ribosomal RNA genes show a high degree of functional consistency and occur in all organisms with different degrees of sequence variations. The sequence analysis of 16S

rRNA gene has been used widely to examine the phylogenetic relationship of species and strains (Woese, 1987). In an exhaustive study, the 16S rRNA sequences have been analysed to determined the relationships of species in the family *Pasteurellaceae* (Dewhirst *et al.*, 1992). The area of DNA sequencing techniques has been developed with automation, computer generated sequences, availability of computer programmes for handling of larger sequences and the internet which provides instant access to common computer programmes and leading databases like the Genbank. With these new developments, rRNA sequence analysis will be a better candidate for studying strain variation among species. 16S rRNA sequence analysis has shown a close relationship among *H. somnus* and *A. seminis* (Dewhirst *et al.*, 1992).

1.4.4 Amplification of DNA by PCR

Any identification and typing system related to nucleic acid depends on the quantity and quality of the nucleic acid preparation. PFGE and hybridisation procedures depend on the availability of nucleic acids in the sample and these procedures can be readily applied only to microbes that can be grown in the laboratory. There are some bacteria and many viruses that are difficult to propagate artificially. This problem can be overcome by amplification of nucleic acids.

In early studies, amplification of nucleic acid was done with the Klenow fragment of *E. coli* DNA polymerase I, which is a heat labile enzyme and had to be added in every cycle after each denaturation step. Thus, it was a laborious and time consuming process. The powerful nucleic acid amplification system, PCR was invented by Seiki *et al.*, (1985) for Cetus Corporation, following the discovery of thermostable *Taq* DNA polymerase. PCR is capable of producing multiple copies of specific nucleic acid regions quickly and exponentially, including non-coding regions of DNA as well as particular genes. Since it is easier to amplify shorter sequences, most applications choose a target length of <2 kb but target sequences of up to 10 kb have been amplified (Jeffreys *et al.*, 1988). The PCR and its applications have been reviewed by several authors (Schochetman *et al.*, 1988; White *et al.*, 1989; Coote, 1990; Erlich and Arnheim, 1992; Henson and French, 1993).

1.4.4.1 The principal and technical details of PCR

PCR is an enzymatic method of exponentially amplifying, in each cycle, a specific preselected or randomly-selected fragment of DNA (**Figure 1.1**). The amplification reaction is catalysed by thermostable *Taq* DNA polymerase and the other components are

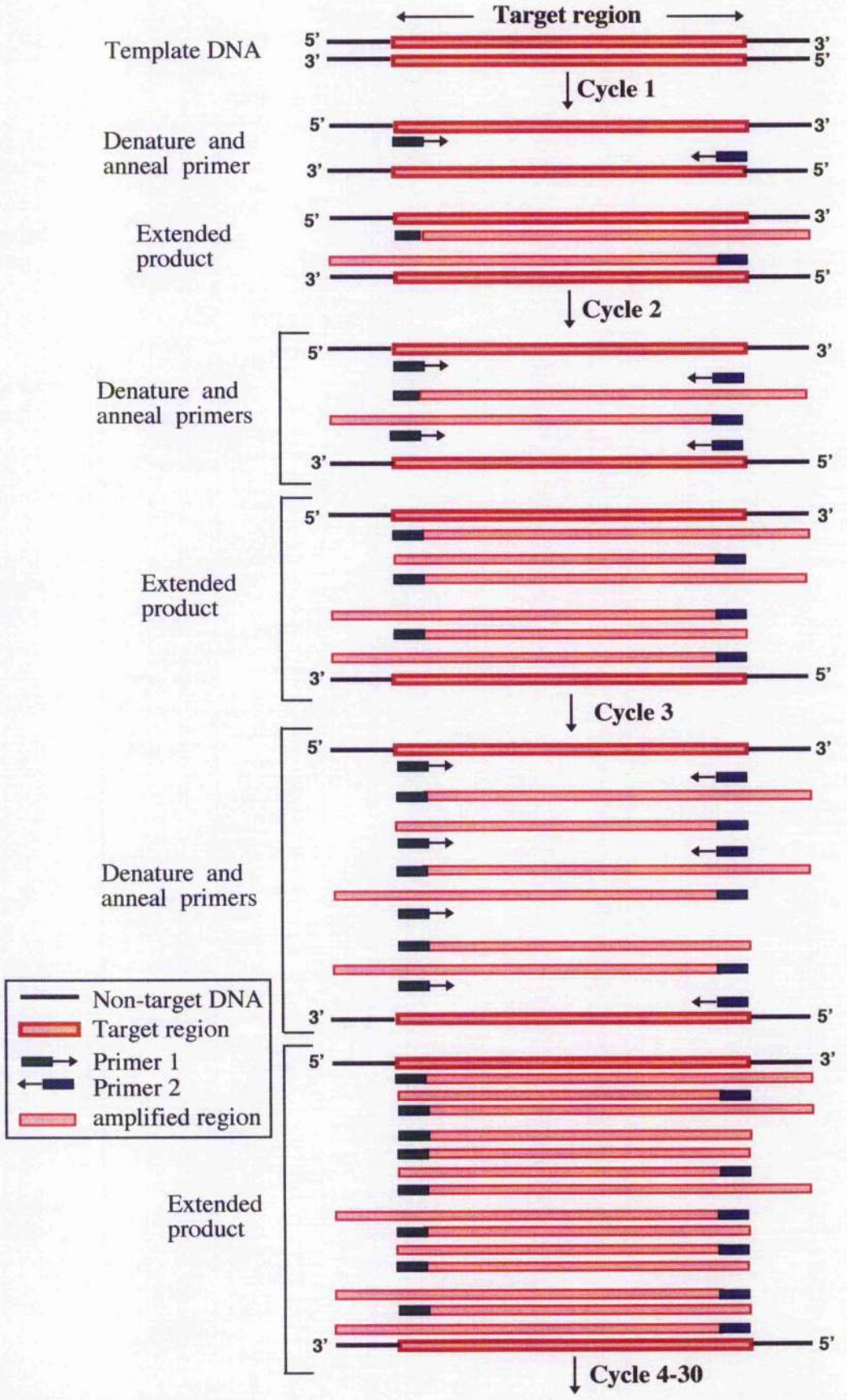

Figure 1.1 *Schematic diagram of exponential amplification by DNA polymerase*

two synthetic oligonucleotide primers, the four standard deoxyribonucleoside triphosphates, template DNA and a buffered reaction medium.

Each amplification reaction has three steps (**Figure 1.1**). The duplex template DNA strands are separated by a heating (usually between 92-94 ^{o}C) step called **melting** or **denaturation**, then the temperature is reduced to a such level that the primers bind specifically with the denatured single DNA strand (**annealing**) and then the temperature is increased to the optimum level (usually 72 ^{o}C) for the activity of the polymerase enzyme, which catalyses primer elongation (**extension**).

1.4.4.2 Reaction components of PCR

Template DNA. Any PCR begins with the preparation of nucleic acid. Theoretically, one copy of the target template is needed, but in practice 10 ng-100 µg of DNA is used to improve the quality of the product. Crude preparation of DNA is usually adequate for PCR, but the presence of large quantities of EDTA should be avoided. This chelates with Mg^{2+} which is essential for the activity of *Taq* polymerase. PCR with purified DNA and crude extracts, like boiled whole cells have produced similar patterns (Corney *et al.*, 1993; Woods *et al.*, 1993; Appuhamy *et al.*, 1997). Micheli *et al.* (1994), however, reported that the reproducibility of PCR may be greatly affected by the quality of DNA isolated and showed that DNA wound to a glass rod was superior to DNA from a centrifuged pellet. However, reproducible fingerprints have been obtained with both extracted chromosomal DNA and whole cell preparations (Kerr, 1994).

Primers. The various procedural variation of the PCR technique requires the use of different types of primers. Primers should be designed in order to achieve the highest specificity and efficiency. Concepts for PCR primer design have been outlined by Dieffenbach *et al.* (1993). The primers for PCR amplification are synthetic oligonucleotides which may be 8-30 nucleotides long and are usually designed by reference to DNA sequence databases. Care has to be taken not to have complementary sequences in each primer particularly at the 3' ends in order to avoid primer-dimer formation. Ideally, both primers should have the same or similar melting temperatures. The ideal primer should contain 50% G+C content. A computer programme has been design for selection of oligonucleotide primers for PCR (Lowe *et al.*, 1990). Each pair of primers must act in concert and must be relatively specific for their binding sites.

DNA polymerase enzyme. Saiki *et al.* (1985) discovered the thermostable DNA polymerase (*Taq*) from *Thermus aquaticus*, a thermophilic eubacterium found in hot

springs and water heaters. The PCR reaction is catalysed by this DNA polymerase and its activity will determine the yield and specificity of target products. The processing activity of *Taq* DNA polymerase is 35-100 nucleotides per second at 70-80 °C (Newton, 1995) and it does not have a 3'-exonuclease activity (Innis *et al.*, 1988). The half-life of *Taq* DNA polymerase in PCR reaction buffer is 130 min at 92.5 °C and 40 min at 95 °C. The efficiency of *Taq* polymerase is increased when PCR buffer is replaced with reverse transcriptase buffer (Krawetz *et al.*, 1989) and the gene 32 protein of the phage T_4 has improved the yield of long amplified DNA fragments at least ten-fold by enhancing *Taq* DNA polymerase activity and accuracy (Schwarz *et al.*, 1990). Bielawski *et al.*, (1995) showed that the gene 32 protein also prevents non-specific primer annealing. It has been reported that the array of DNA products of PCR may be greatly affected by the concentration of *Taq* polymerase enzyme (Brikun *et al.*, 1994). **Table 1.4** shows the components of a typical PCR mixture.

1.4.4.3 Reaction conditions of PCR

PCR is a cyclic reaction which amplifies the target sequences exponentially in each cycle. The typical PCR amplification has three stages i.e. an initial cycle with a relatively long and high temperature denaturation step in order to complete denaturation of template DNA, then normal cycles and finally a step with a relatively long extension time for the completion of partially amplified PCR products. The initial denaturation is usually at 94 °C for 2-4 min. Bielawski *et al.*, (1995), however, showed that omission of this initial long denaturation step increased the band intensity and reduced the level of smearing. A typical PCR protocol involves denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 2-3 min with 25 cycles (Coote, 1990). However, these protocols differ from experiment to experiment and application to application. For example, random amplified polymorphic DNA (RAPD) PCR is usually done in 45 cycles with denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min extension at 72 °C for 2 min (Williams *et al.*, 1990) and in contrast, REP-PCR is usually performed with 30 cycles in denaturation at 90 °C for 30 sec, annealing at 40 °C for 1 min and extension at 65 °C for 8 min with initial denaturation at 95 °C for 7 min and final extension at 65 °C for 16 min (Versalovic *et al.*, 1991). Schweder *et al.*, (1995) showed that the ramp time (transition interval between temperature differences) has a great effect on the reproducibility of RAPD. The ramp is determined by the flow of cooling water or air in the thermal cycler and its temperature, which in turn depends on the ambient temperature.

Table 1.4 ***The components of a typical PCR reaction***

Component	Concentration
DNA template	10ng-100µg/ml
dNTP (each nucleotide)	200 µg
Primer 1	0.1-1.0 µM
Primer 2	0.1-1.0 µM
Taq polymerase	1 unit
KCl	50 mM
Tris-HCl (pH 8.4 at 25 °C)	10 mM
$MgCl_2$	2.5 mM
Gelatin	100 µg/ml
Nonidet-40	0.05% (v/v)
Total volume	25-100 µl

1.4.4.4 Optimisation of PCR

As the PCR is highly sensitive technique, its performance is adversely affected with a slight variation of the components of the reaction mixture and the reaction conditions. Each new application of PCR may require optimisation. Inadequate functioning of PCR may be observed as no detectable product or a low yield of the target band, high level of background due to mispriming or misextension of the primers, the formation of primer-dimers. The concentrations of the polymerase enzyme, deoxynucleotide triphosphate and magnesium ion are reported as critical components to be optimised in the PCR reaction (Innis and Gelfand, 1990) A simple procedure for optimisation of PCR based on orthogonal array has been applied to avoid laborious steps (Cobb and Clarkson, 1994).

1.4.4.5 Visualisation of PCR products

Generally, the PCR products are visualised under UV light after electrophoresis in agarose or polyacrylamide gels stained with ethidium bromide (Sambrook *et al.*, 1989). Several alternative methods have been sought. The PCR products were detected after hybridisation with ^{32}P-labeled probe (Ochman *et al.*, 1988) or alkaline phosphatase labled probe (Cano *et al.*, 1993). With this alkaline phosphatase system, it has been possible to detect 1-10 colony forming units of *Salmonella* species. In another method, for the detection of PCR products, a fibre-optic biosensor has been used (Strachan and Gray, 1995). The PCR products were amplified with biotin and fluorescein-labelled primers. The biotin was used to immobilise single stranded PCR products to fibres coated with streptavidin. When the fluorescein-labelled complementary PCR product hybridised to the fibre, a voltage was generated and detected by a sensor. In another technique, PCR products were analysed quantitatively by an automated method that combines capillary-gel electrophoresis for high efficiency seperation and laser induced fluorescence for highly sensitive detection (Lu *et al.*, 1994). Although, these methods have increased the sensitivity of detection, they are technically demanding and time consuming.

1.4.5 Procedural variations to PCR

The basic PCR technique has been adopted to suit specific applications by changing its components and procedures.

Nested PCR. This method uses several sets (usually two) of primers in different steps. The first primer set amplifies a large product and the subsequent primers target this

product as the template DNA. The final product therefore is shorter than the initial product. By this method, the level of specificity is increased and there is an increase in amplification efficiency by minimising non-specific primer annealing but it is obviously more laborious than single step PCR.

Multiplex PCR. In this system, multiple pairs of primers are used to amplify many DNA products from several target DNA segments in the same reaction.

Reverse transcription-PCR (RT-PCR). In 'normal PCR,' the template DNA is added to the reaction mixture in order to get the desired amplified fragment. In RT-PCR, the template DNA is transcribed from a target RNA sequence with reverse transcriptase enzyme or DNA polymerase having reverse transcriptase activity.

Inverse PCR. Inverse PCR has been used to synthesis the DNA flanking a known sequence. The outward directed primers of inverse PCR amplify upstream and/or downstream flanking regions on either side of the known sequence (Ochman *et al.*, 1988).

1.4.6 Applications of PCR in microbiology

PCR has been applied to many fields i.e. research, disease diagnosis, forensic use etc. For example, PCR has been applied to prenatal diagnosis of genetic diseases, determination of the sex of human embryos fertilised *in vitro*, the analysis of ancient bone samples and in forensic laboratories the DNA from hairs has been amplified. The uses of PCR relevant to microbiology has been discussed in detail by Coote (1990). In addition to molecular biology, gene manipulation, cloning and sequencing, PCR can be used not only to detect but also to type microorganisms (Coote, 1990; Welsh and McClelland, 1990; Williams *et al.*, 1990; Versalovic *et al.*, 1991; Tigano-Milani *et al.*, 1995) as in the present investigation.

1.4.7 Fingerprinting of bacteria by PCR

1.4.7.1 RFLP analysis of PCR products

PCR has been combined with restriction endonuclease digestion of PCR products which are then resolved on agarose or polyacrylamide gels to produce RFLPs. This system has been successfully applied to differentiate strains of rickettsiae by amplifying the citrate

synthetase gene (Regnery *et al.*, 1991). The advantages of this system are that small quantities of chromosomal DNA are sufficient and the procedure takes only a short time.

1.4.7.2 Randomly Amplified Polymorphic DNA (RAPD) PCR

Welsh and McClelland (1990) and Williams *et al.* (1990) reported simultaneously that organisms could be typed using PCR fingerprints generated with primers having random sequences. The application of RAPD typing in microbiology has been reviewed by Power (1996). This method has been termed arbitrarily-primed PCR (AP-PCR) or randomly amplified polymorphic DNA (RAPD-PCR). For conventional PCR, knowledge of target sequence is a prerequisite and that drawback has been eliminated with RAPD-PCR. The basis of the method is that a single primer combined with two cycles of PCR at low stringency and many cycles at high stringency generate a discrete and reproducible set of amplification products characteristic of particular genomes. The theory for this method is that at a sufficiently low temperature a primer can anneal to many sequences with a variety of mismatches. Some of these sequences will be within a few hundred base pairs of each other and on opposite strands, such that the intervening sequences will be amplified by PCR (Welsh and McClelland, 1990). The primers used for RAPD have varied in the number of nucleotides: 20 (Welsh and McClelland, 1990); 10-12 (Williams *et al.*, 1990); and as short as five (Caetano-Annolles *et al.*, 1991).

The applications of RAPD in microbiology have been reviewed (Kerr, 1994). It has been used in different investigations. Different phyletic groups corresponding to geographical distribution of isolates of *Borrelia burgdorferi* have been shown using RAPD (Welsh *et al.*, 1992). RAPD has been used to identify virulent pathotypes of the phytopathogenic fungus *Leptosphaeria maculans* (Goodwin and Annis, 1991) and for the analysis of the pedigree of K12 strains of *E. coli* (Brikun *et al.*, 1994). The RAPD technique has been used with a capillary air thermal cycler for differentiation of *Listeria* species and *Listeria monocytogenes* serotypes (Black *et al.*, 1995). This capillary air thermal cycler method has an advantage over conventional metal heat block thermal cyclers in that the temperature changes are very rapid.

Meunier and Grimont (1993) have investigated the reproducibility of RAPD fingerprinting. They found that different brands of *Taq* DNA polymerase have drastic effects on the reproducibility of banding patterns of RAPD and that the cause of this variation was the buffer recommended by the manufacturer, not the *Taq* DNA polymerase. They also found that make of thermal cycler affected the reproducibility of the RAPD pattern. Van Belkum and Meis (1994) reported that RAPD was a good PCR fingerprinting

method that gave excellent resolution, a high degree of reproducibility, exquisite sensitivity and extreme versatilty when compared to other typing techniques and that large numbers of strains can be analysed within a short period of time. In a reply to van Belkum and Meis (1994), Arbeit *et al.* (1994) mentioned that RAPD produces some bands by inefficient reactions, resulting in variation in amplification and generation of bands of different sizes and intensity that cause difficulties in comparing profiles. They also reported that there are no standards for the reproducibility of RAPD and the discriminatory power of pulse field gel electrophoresis is higher than RAPD. On the other hand, it has been reported that RAPD typing is far more sensitive than multilocus enzyme electrophoresis typing for discriminating between related strains of a species (Wang *et al.*, 1993) and a comparative study of ribotyping, PFGE and RAPD has shown that the discriminatory power and analysis of patterns is higher but time-consuming in PFGE than RAPD (Chachaty *et al.*, 1994). In contrast, Vila *et al.* (1994) reported that ribotyping and RAPD produce a similar discriminatory power for typing *Acinetobacter baumannii*, although RAPD has additional advantages of speed and simplicity. In an another study, van Belkum *et al.* (1994) showed that RAPD is a reliable and reproducible method for genotyping of non-capsulate strains of *Haemophilus influenzae* and RAPD fingerprinting is easier to interpret than, but has similar discriminatory power to, RFLP analysis. As PCR is highly sensitive to its components (Innis and Gelfand, 1990) the RAPD has also shown the dependency on all those parameters. The standardisation of the RAPD method may solve these problems (Power, 1996).

1.4.7.3 Repeat element PCR

Repetitive extragenic palindromic PCR (REP-PCR) and enterobacterial repetitive intergenic consensus (ERIC-PCR). Intergenic repeated sequences have been reported primarily in the enteric bacteria *E. coli* and *Salmonella typhimurium*. These sequences contain highly conserved inverted repeats which are of two types and do not share significant homology. Repetitive extragenic palindromic elements (REP) are one type and the other is enterobacterial repetitive intergenic consensus (ERIC) (Towner and Cockayne, 1993).

The functions of these elements are not well documented. It has been suggested that they are involved in stabilising mRNA (Newbury *et al.*, 1987), transcription termination and translational coupling between genes (Stern *et al.*, 1988), homologous recombination (Shyamala *et al.*, 1990), chromosomal domain organisation and binding of HU proteins, DNA gyrase and DNA polymerase I (Higgins *et al.*, 1982). The REP units are 38 bp long (Versalovic *et al.*, 1991) with a estimated number of copies of the REP sequence on the

chromosome of *E. coli* greater than 500, equivalent to between 0.5-1% of the total genome (Stern *et al.*, 1984). The ERIC element contains 126 bp and does not appear to be related to the REP sequences and the copy numbers are variable (Versalovic *et al.*, 1991).

Versalovic *et al.* (1991) produced primers targeting these REP and ERIC sequences to amplify the regions between two sequences by PCR and revealed that inter-REP or inter-ERIC distances and patterns are specific for bacterial species and strains and amplification is limited to adjacent repeat elements within the limitation of polymerase extension (**Figure 1.2**). Differences in band sizes presumably result from polymorphisms in the distance between REP or ERIC sequences in different genomes (Kerr, 1994). There is no requirement for genus or species-specific primers for REP- or ERIC-PCR. It has now been applied to characterisation of many of bacterial species and the knowledge of the DNA sequence of the bacteria is not necessary. Reproducible fingerprints have been produced with both extracted chromosomal DNA and whole cell preparations (Judd *et al.*, 1993; Woods *et al.*, 1993; Rodriguezbarradas *et al.*, 1995).

BOX repeat element PCR. The BOX repeat element was initially found in the chromosome of *Streptococcus pneumoniae*. These BOX elements consist of three subunits i.e. BoxA, BoxB and BoxC which are 59, 45 and 50 nucleotides long, respectively. There is no relationship between these elements and the REP and ERIC units. The function of the BOX elements may have a connection with genetic transformation or be involved in virulence of *S. pneumoniae* as these units are located in the vicinity of genes responsible for those functions (Martin *et al.*, 1992). The primers targeting these BOX elements have been used to fingerprint not only *S. pneumoniae* strains (van Belkum *et al.*, 1996) but also plant pathogens (Louws *et al.*, 1995).

1.4.7.4 PCR-ribotyping

All organisms depend on the function of rRNA genes for their survival and they show a high degree of conservation in all organisms with different rates of sequence variation. In prokaryotes, the rRNA genetic loci contain the genes for all three rRNA species, 16S, 23S and 5S genes and may contain one or more tRNAs genes (**Figure 1.3**). The rRNA genes are separated by spacer regions which exhibit a large degree of sequence and length variation at the level of genus and species. Within a single genome there are frequently multiple rRNA genetic loci; spacer regions found within these loci also show a significant degree of variation in length and sequence. This diversity is due in part to variations in the number and type of tRNA sequences found within the spacers. Most bacteria contain between 2 and 11 rRNA gene copies per genome, while the intergenic

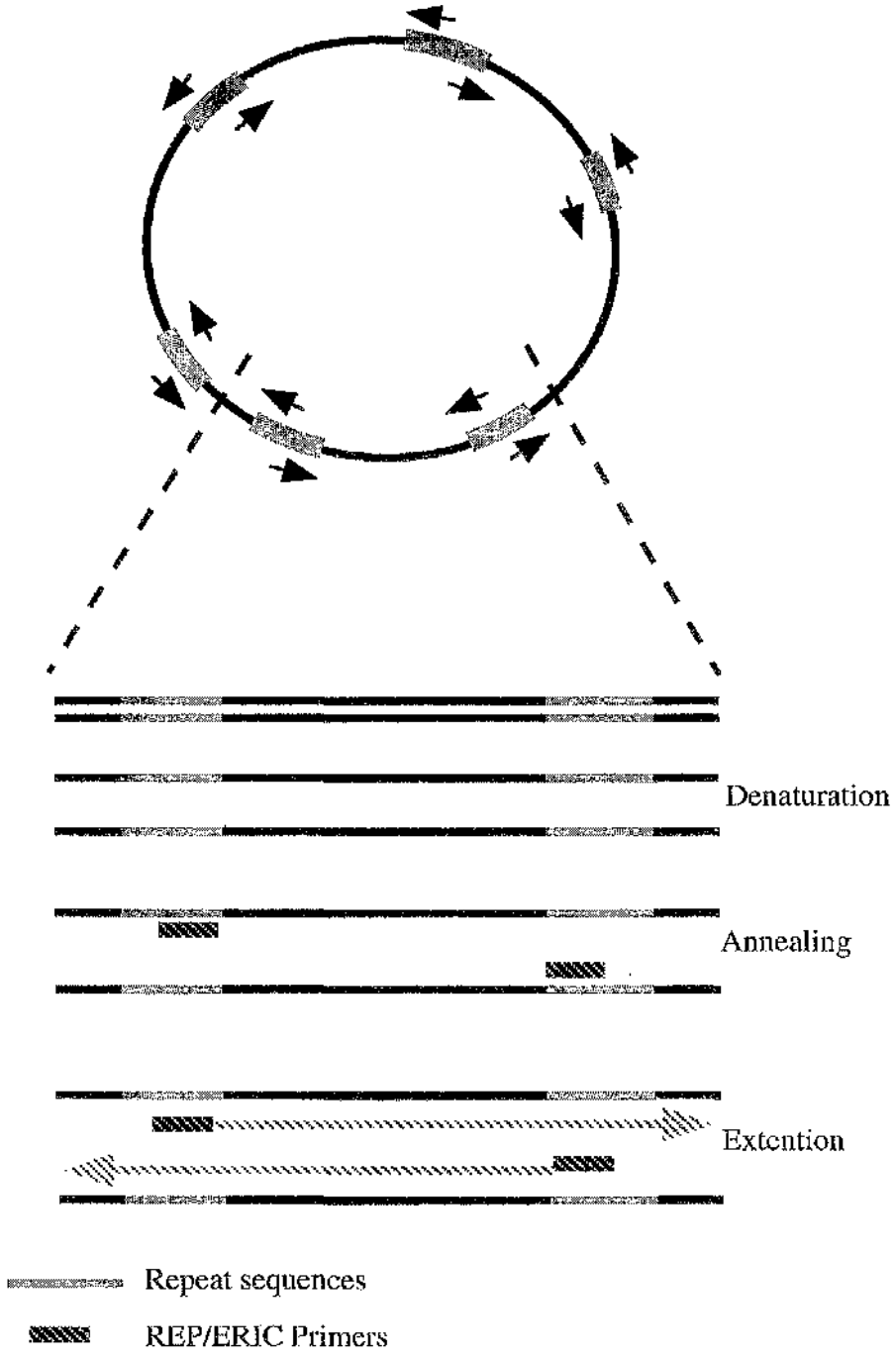

Figure 1.2 ***Schematic diagram of repeat sequences in the bacterial genome and their amplification by REP and ERIC PCR***

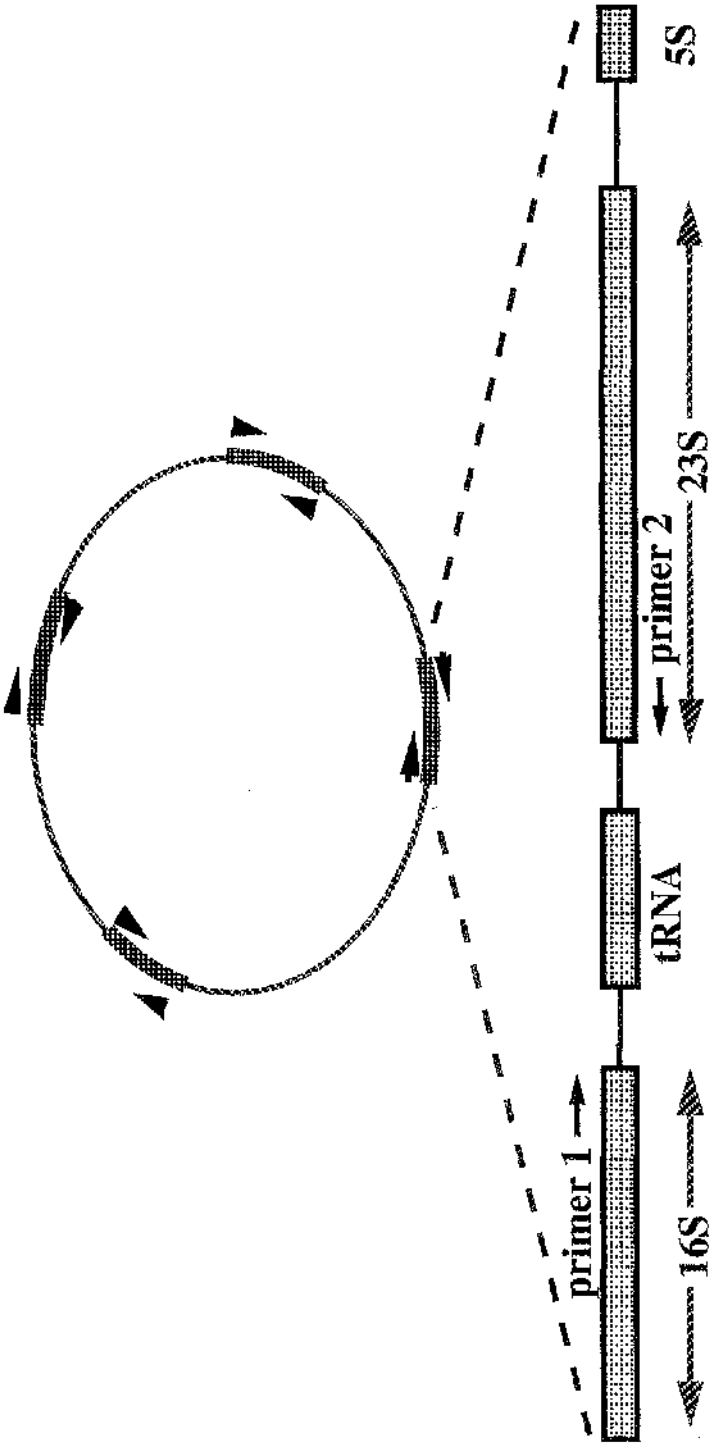

Figure 1.3 *Schematic diagram of structure and distribution of RNA operons in the bacterial genome and the target of the primers for amplification of 16S and 23S spacer region by PCR-ribotyping*

spacer regions between the 16S and 23S rRNA genes encode various tRNAs and contain several direct repeat sequences in non-coding regions of the gene clusters (Gurtler and Stanisich, 1996).

Barry *et al.* (1991) showed that this length and sequence polymorphism of spacer regions of rRNA loci can be used to differentiate prokaryotic species. Subsequently, this 16S and 23S spacer region has widely been used for identification and characterisation of bacteria by PCR. As it involves both PCR and rRNA operons, the name **PCR-ribotyping** has been used for this method but Vaneechoutte (1996) proposed the name **repeat-length polymorphism analysis** as the spacer region of 16S and 23S rRNA genes of ribosomal operons vary in intra- and extra-species level.

For PCR-ribotyping studies, four or six highly conserved regions of the 16S and 23S genes have been targeted respectively, in different studies (Gurtler and Stanisich, 1996). The number of bands in fingerprints of PCR-ribotyping is comparatively few and sometimes only a single band is produced and showed no strain variation within the species (Vaneechoutte, 1996). The additional step of a restriction digest of those PCR products has been shown to be useful for differentiation of strains in such cases (Ryley *et al.*, 1995). Using consensus sequence tRNA gene primers, Welsh and McClelland (1991) have generated reproducible fingerprints from many microorganisms. This PCR-based ribotyping has many advantages over probe hybridisation, because probe hybridisation requires more technical skill and more time.

1.4.7.5 Amplified restriction fragment polymorphism (AFLP)

This novel DNA fingerprinting technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps: restriction of the DNA and ligation of oligonucleotide adapters (they provide specific complementary regions for primer annealing), selective amplification of sets of restriction fragments and gel analysis of the amplified fragments. The advantange of this method is that knowledge of DNA sequences is not necessary. The method allows the specific co-amplification of high numbers of restriction fragments. The number of fragments that can be analyzed simultaneously, however, is dependent on the resolution of the detection system, typically 50-100 restriction fragments are amplified and detected on denaturing polyacrylamide gels (Vos *et al.*, 1995). The disadvantages of the method is that it is labour intensive and reproducibility is difficult as it depends on uniform adaptor ligation, so it may not be considered as the first method of choice (Janssen and Dijkshoorn, 1996).

1.4.8 Comparative studies on different typing methods

The results of ribotyping, PFGE and RAPD obtained for *Clostridium difficile* strains have shown that the PFGE had highest discrimination while ribotyping showed the least. The RAPD was easier to perform but the results were complex and difficult to analyse (Chachaty *et al.*, 1994). Five molecular techniques, monoclonal antibody-based serotyping and serosubtyping, PFGE, MEE, ribotyping and PCR-ribotyping-RFLP have been compared for subtyping of *Neisseria meningitidis* serotype B isolates from the United States and north western Europe (Swaminathan *et al.*, 1996). The PFGE, MEE and ribotyping showed greater discriminatory abilities (>99%) than monoclonal antibody-based serotyping or PCR-ribotyping-RFLP. The discriminatory power of ERIC-PCR and PFGE for *Flavimonas oryzihabitans* was found to be the same but ERIC-PCR was better due to its speed and simplicity (Liu *et al.*, 1996). Struelens *et al.* (1993) reported that the discriminatory power of ERIC-PCR is less than that of PFGE. The discriminatory power of MEE has been compared with REP-PCR. Woods *et al.* (1992) reported that both techniques had similar discriminatory power, whereas, Versalovic *et al.* (1993) showed that REP was less discriminatory than MEE with *Streptococci*, explained possibly by the lower incidence of REP sequences in *Streptococci*. Versalovic *et al.* (1991) compared the discriminatory power of REP and ERIC with various Gram-negative and Gram-positive bacteria and found that in general REP is less discriminatory than ERIC. However, Giesendorf *et al.* (1994), with Campylobacter species, showed that both REP and ERIC have similar discriminatory power. In a separate study, AP-PCR, ERIC-PCR and PFGE showed 100% discrimination for 23 epidemiologically unrelated *Burkholdaria cepacia* isolates but only 52% discrimination was shown by PCR-ribotyping (Liu *et al.*, 1995) and their conclusion was that ERIC-PCR seemed to be more reproducible and discriminatory.

1.4.9 Identification and detection of bacteria by PCR

Nucleic acid amplification by PCR has been applied to the detection and identification of microbes and is becoming widely used for detection for diagnostic purposes in clinical research and diagnostic laboratories (Whelen and Persing, 1996; Vaneechoutte and VanEldere, 1997). The PCR allows identification of low amounts of DNA and is highly specific since the amplification depends on the use of primers with sequences which are complimentary to the DNA molecule to be amplified. The PCR is not hindered by the presence of DNA from other sources and therefore allows the selective amplification of one DNA molecule with a predefined sequence in the presence of large amounts of other nucleic acids. The ability to specifically amplify DNA by PCR from low numbers of bacteria, as

well as its simplicity, rapidity and reproducibility offers advantages over the conventional cultural and phenotypic methods for identification.

The design of primers were based on different methods. Most of them were targetting species specific genes such as *tox*A of *P. multocida* (Nagai *et al.*, 1994; Lichtensteiger *et al.*, 1996), *fla*A gene of *Listeria* (Gray and Kroll, 1995), 16S gene of *Haemophilus ducreyi* (West *et al.*, 1995) and *Brucella* (Romero *et al.*, 1995), urease subunits gene of *Helicobacter pylori* (Furuta *et al.*, 1996), a membrane protein gene of *Chlamydia psittaci* (Domeika *et al.*, 1994) and 18S gene of *Aspergillus* (Yamakami *et al.*, 1996). A specific oligonucleotide probe which is complimentary to a 16S rRNA sequence was used as one of the primers in a RNA reverse transcription and cDNA PCR amplification assay (RT-PCR) for the detection of *Helicobacter* spp (Engstrand *et al.*, 1992). This method has increased the sensitivity up to 50-fold when compared with conventional PCR. PCR amplification of the 16S-23S rRNA spacer region has been suggested as the basis of a universal bacterial identification and typing system (Barry *et al.*, 1991; Jensen *et al.*, 1993; Gurtler and Stanisich, 1996). This method has been adopted by Smart *et al.* (1996) for detection of Phytoplasmas and by Tilsala-Timisjarvi and Alatossava (1997) for detection of lactic acid bacteria.

1.4.10 Problems with PCR

This technique suffers from several shortfalls (Vaneechoutte and VanElderc, 1997). The major problem is false positive results. This is due to contamination. The source of contamination may be from other samples or products of previous amplifications (carryover). Processing of negative samples along with each sample preparation is the way of detection of false positive results due to contamination (Vaneechoutte, 1996).

The other problem of the use of PCR as a diagnostic test is the false negative results. Poor amplification due to intrinsic errors may be one reason for this type of result and optimisation of reaction mixture and reaction conditions is important. The presence of polymerase inhibitors in the samples can yield false negative results. Traces of phenol from extracted DNA is a known inhibitor for PCR. This method has been widely applied to detect organisms from different biological materials, such as *Streptococcus pneumoniae* from blood (Zhang *et al.*, 1995), *Salmonellae* in faeces (Widjojoatmodjo *et al.*, 1992), *Chlamydia psittaci* and *E. coli* in bovine semen (Domeika *et al.*, 1994; Gradil *et al.*, 1994). The inhibitory components that are present in these biological materials had a great effect on the perfomance and the sensitivity of the PCR assays (Panaccio *et al.*, 1994).

Different attempts have been made to overcome this inhitory effect of biological materials for PCR. The chelating compound, Chelex 100 (Bio-Rad Laboratories, Hemel Hampstead, UK) is a styrene divinylbenzene copolymer that has high affinity for polyvalent metal ions. It has been suggested that the presence of Chelex 100 during boiling of samples prevents the degradation of DNA by chelating metal ions that may act as catalysts in the shearing of DNA at high temperature in low ionic strength solutions (Walsh *et al.*, 1991). Chelex 100 5% (w/v) suspension was found to be the optimal concentration for preparation of DNA template by boiling (de Lamballerie *et al.*, 1992) and it has successfully been used for DNA template preparation from *Chlamydia psittaci* from bovine semen (Domeika *et al.*, 1994). FoLT PCR is an efficient alternative method based on the use of formamide for amplification of DNA directly from whole blood and that uses *Tth* polymerase as the enzyme for the PCR. The porphyrin compounds in blood are known inhibitors for *Taq* polymerase but not for *Tth* polymerase. The other advantages of this FoLT PCR are minimum number of manipulations and the whole procedure can be done in a single tube (Panaccio *et al.*, 1994). Application of immunocapture methods are another way for removal of inhibitory components. Microfuge tubes were pre-coated with anti-histone antibodies which can capture DNA from any lysed cells. The potential inhibitors of PCR can be removed by washing and the PCR is performed in the same tube. This method has been successfully applied to detect *Plasmodium falciparum* DNA in human blood (Panaccio *et al.*, 1994).

Proteinase K digestion of the sample is an another method that has been applied to remove inhibitory components from the sample. Inhibitory components of bull semen have been removed by this method to detect *Clamydia psittaci* by PCR (Domeika *et al.*, 1994). This method was also found to be useful for detection of bacterial DNA from faecal samples (Panaccio *et al.*, 1994).

Another reason for false negatives may be low numbers of 'target' cells present in the sample. Usually, most clinical samples contain a limited number of organisms. This fact is in turn aggravated in PCR as microlitre volumes are used in a reaction so reducing the probability of DNA templates being present in the reaction.

The establishment cost of PCR is also high. The equipment from micropipettes for minute volumes (e.g. 0.5-2 µl) to thermocyclers are expensive. The consumables like DNA molecular markers and DNA polymerase enzyme are expensive but essential components. The visualisation of the results needs electrophoresis apparatus and a UV transilluminator. The extreme sensitivity of PCR can also lead to considerable problems. Although, it is an advantage of PCR, the ability to detect nucleic acids from dead as well as viable organisms can give a false positive reaction for PCR. Therefore it is necessary to perform PCR under

extreme cleanliness and with rigorous controls. Minute changes in reaction components can lead to severe changes in results. For example, doubling of the magnesiun ion concentration may change the total amount of product produced by several orders of magnitude (Giovannoni, 1991) and changes of *Taq* polymerase can also alter results drastically. The relatively high error rate of *Taq* polymerase is an another problem. Base substitutions occur at about one in every 9000 bp and frameshifts at about one in every 40000 bp. Although PCR can now be semi-automated, the technique still requires a certain amount of technical skill and some specialised equipment to perform a DNA amplification successfully (Towner and Cockayne, 1993).

OBJECTIVES OF RESEARCH

The identification and differentiation of *H. somnus*, *H. ovis* and *A. seminis* is difficult as they are fastidious, slow growing organisms and show similar cultural and biochemical properties. Reports of isolation of these pathogens are becoming increasingly common not only in Scotland but also worldwide in the cattle and sheep farming industries. The main objective of this study was the development of PCR-based fingerprinting techniques as rapid and reproducible methods to differentiate these species and their strains. The PCR typing methods were compared with existng traditional methods such as conventional biochemical tests, plasmid profile analysis and antibiotic sensitivity patterns for their utility. A further goal of this work was to develop a PCR-based diagnostic test as a rapid and reliable method to identify these bacteria in primary culture and also to detect them directly in clinical samples.

2. MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

A total of 72 bacterial isolates (test isolates) were used. They belonged to three species of bacteria, namely *Haemophilus somnus*, *Histophilus ovis* and *Actinobacillus seminis* and a description of each isolate is given in the **Tables 2.1, 2.2** and **2.3**, respectively. As the taxomomy of these three species is not clear, isolates showing properties similar to those described by Humphrey and Stephens (1983) from cattle and sheep are referred to as *Haemophilus somnus* and *Histophilus ovis* respectively. Isolates which showed properties similar to those described by Hajtos *et al.* (1987) are referred to as *Actinobacillus seminis* throughout this thesis.

2.1.1 *Haemophilus somnus*

Twenty nine isolates were obtained during the study. The type strain of *H. somnus* (THs), ATCC #43626, was obtained from the American Type Culture Collection although its origin is not traceable. Other *H. somnus* strains were field isolates and they were kindly supplied by Dr. D. J. Taylor, Department of Veterinary Pathology, University of Glasgow and by Veterinary Investigation Officers of the Scottish Agricultural College Veterinary Services (SACVS) throughout Scotland (**Table 2.1**). These were of both respiratory and reproductive origin and included isolates from both diseased and clinically normal animals. Isolates SA21, SA22 and SA23 were recovered from different bulls from the same herd that had a history of subnormal fertility. Isolates V3, V8, X1 and X4 were isolated from slaughterhouse material as detailed in **section 2.5**.

2.1.2 *Histophilus ovis*

Nineteen isolates of *Histophilus ovis* were included in the study (**Table 2.2**). Isolates SA08 and SA16 were kindly provided by the Veterinary Investigation Officer of SACVS, Ayr, Scotland. Isolate SA24 was used as the reference isolate (Low and Graham, 1985) as there was no type strain available in any of the major culture collections and SA24 was the first recorded isolate from the UK. This and the remainder of the *H. ovis* isolates were generously supplied by Dr. J. C. Low, Veterinary Investigation Officer of SACVS, Edinburgh, UK. All of these bacteria were isolated from semen of either clinically normal rams or rams with fertility problems, except for SA56, SA57 and SA58 which were isolated from different ewes in the same flock.

Table 2.1 *Description of H. somnus isolates*

Isolate	Source	Site of isolation	Disease status	Geographic origin	Origin†
THs*	Bovine	No record	No record	No record	ATCC
SA01	Bovine	No record	No record	No record	DJT
SA02	Bovine	No record	No record	No record	DJT
SA03	Bovine	No record	No record	No record	DJT
SA04	Bovine	Lung	Pneumonic	Dumfries	SACVS
SA05	Bovine	Lung	No record	Dumfries	SACVS
SA06	Bovine	Lung	Pneumonic	Ayr	SACVS
SA07	Bovine	Lung	Pneumonic	Aberdeen	SACVS
SA11	Bovine	semen	Normal	St. Boswells	SACVS
SA12	Bovine	semen	Normal	St. Boswells	SACVS
SA13	Bovine	Lung	Pneumonic	Aberdeen	SACVS
SA14	Bovine	Lung	Pneumonic	Aberdeen	SACVS
SA15	Bovine	Lung	Pneumonic	Ayr	SACVS
SA17	Bovine	Lung	Normal	Aberdeen	SACVS
SA19	Bovine	Lung	Pneumonic	Aberdeen	SACVS
SA20	Bovine	Lung	Pneumonic	Aberdeen	SACVS
SA21[a]	Bovine	prepuce	Subfertile	Glasgow	DJT
SA22[a]	Bovine	prepuce	Subfertile	Glasgow	DJT
SA23[a]	Bovine	prepuce	Subfertile	Glasgow	DJT
SA48	Bovine	Lung	Pneumonic	Aberdeen	SACVS
SA49	Bovine	Lung	Pneumonic	Aberdeen	SACVS
SA50	Bovine	Lung	Pneumonic	Aberdeen	SACVS
SA51	Bovine	No record	No record	Edinburgh	SACVS
SA52	Bovine	Vagina	Inflamed	Edinburgh	SACVS
SA68	Bovine	Lung	Pneumonic	Edinburgh	SACVS
V3	Bovine	Vestibular opening	Culled	No record	Own isolate
V8	Bovine	Cervix	Culled	No record	Own isolate
X1	Bovine	Cervix	Culled	No record	Own isolate
X4	Bovine	Vagina	Culled	No record	Own isolate

† ATCC: American Type Culture Collection; DJT: Dr. D. J. Taylor, Department of Veterinary Pathology, University of Glasgow; SACVS: Scottish Agricultural College Veterinary Services; Own isolates: Isolated in this study (**section 2.5**).

* THs: ATCC type strain.

[a] Isolated from different animals of the same herd that showed subnormal fertility.

Table 2.2 *Description of H. ovis strains*

Isolate	Source	Site of isolation	Disease status	Geographic origin	Origin†
SA08	Ovine	Semen	No records	No records	Ayr
SA16	Ovine	Semen	No records	No records	Ayr
SA24*	Ovine	Semen	Infertile	South Scotland	JCL
SA26	Ovine	Semen	Fertile	Central Scotland	JCL
SA27	Ovine	Semen	Subfertile	North England	JCL
SA28	Ovine	Semen	Subfertile	North England	JCL
SA29	Ovine	Semen	Fertile	No records	JCL
SA44	Ovine	Semen	Fertile	No records	JCL
SA45	Ovine	Semen	No record	South Scotland	JCL
SA46	Ovine	Semen	Fertile	Central Scotland	JCL
SA53	Ovine	Semen	Infertile	Central Scotland	JCL
SA54	Ovine	Semen	Subfertile	Central Scotland	JCL
SA55	Ovine	Semen	Infertile	North Scotland	JCL
SA56[b]	Ovine	Vagina	Subfertile	Central Scotland	JCL
SA57[b]	Ovine	Vagina	Subfertile	Central Scotland	JCL
SA58[b]	Ovine	Vagina	Subfertile	Central Scotland	JCL
SA69	Ovine	Semen	Fertile	South Scotland	JCL
SA72	Ovine	Prepuce	Fertile	Central Scotland	JCL
SA73	Ovine	Semen	No records	North England	JCL

† Ayr: SACVS, Ayr; JCL: Dr. J. C. Low, SACVS, Edinburgh.

* SA24: reference isolate (Low and Graham, 1985).

[b] Isolated from different animals of the same flock that showed subnormal fertility.

2.1.3 *Actinobacillus seminis*

Twenty four isolates of *A. seminis* were obtained for the study (**Table 2.3**). The type strain of *A. seminis* (TAs), NCTC (National Collection of Type Cultures) #10851 and field isolates were kindly provided by Dr. J. C. Low. The type strain TAs originated from Australia (Baynes and Simmons, 1960) and all the other isolates except X16 were recovered from rams from clinically normal, infertile or diseased animals. Isolate X16 was recovered from slaughterhouse materials as described in **section 2.5**.

2.1.4 Other bacteria

The following bacterial strains were also included for comparative study. Two strains of *Pasteurella multocida* serotype D, two strains of *P. multocida* serotype B:2, two strains of *P. haemolytica* and one strain of *P. trehalosi* were kindly provided by Dr. R. Parton and *Escherichia coli* strains K12 and DH5α containing plasmid pUC19 were kindly provided by Dr. J. G. Cootc, Division of Infection and Immunity, University of Glasgow. *Actinobacillus actinomycetemcomitans* type strain NCTC 9710 and three Gram-negative pleomorphic bacteria from ram semen samples were provided by Dr. J. C. Low. Five unknown strains isolated in this study from the bovine reproductive tract were also used for specific PCR experiments.

2.2 RAM SEMEN SAMPLES

Fresh ram semen samples and processed samples with added storage solution were kindly provided by Dr. M. J. A. Mylne, Veterinary Officer In Charge, Edinburgh Genetics, SACVS, Edinburgh, UK. The storage solution for semen contained 3.876 g of Tris, 0.533 g of glucose, 2.123 g of citric acid, 16 ml of egg yolk, 5.3 ml of glycerol, 100,000 iu of penicillin, 100 mg of streptomycin and glass distilled water to 100 ml (Evans and Maxwell, 1987). Three parts of this solution were added to one part of raw semen and the mixture was stored in liquid nitrogen. The extent of *A. seminis* contamination of raw semen was determined by spreading 100 µl of 10-fold dilutions of semen samples in sterile distilled water on to BHIBYE agar (**section 2.3.1**) in duplicate. Plates were incubated at 37 ^{o}C for 48 h in a candle jar.

Table 2.3 *Description of A. seminis isolates*

Isolate	Source	Breed of ram	Disease status	Geographic origin	Origin†
TAs*	Ovine	No record	Epididymitis	Australia	NCTC
SA25	Ovine	No record	No record	No record	JCL
SA30[c]	Ovine	Suffolk	Normal	South Scotland	JCL
SA31	Ovine	Suffolk	Subfertile	North Scotland	JCL
SA32	Ovine	Suffolk	Epididymitis	England	JCL
SA33	Ovine	Poll Dorset	Epididymitis	England	JCL
SA34[d]	Ovine	Suffolk	Epididymitis	South Scotland	JCL
SA35	Ovine	Texel	Normal	South Scotland	JCL
SA36	Ovine	Scottish Blackface	Normal	South Scotland	JCL
SA37	Ovine	Suffolk	Epididymitis	South Scotland	JCL
SA38[d]	Ovine	Suffolk	Epididymitis	South Scotland	JCL
SA39	Ovine	Suffolk	Normal	South Scotland	JCL
SA43	Ovine	Texel	Epididymitis	South Scotland	JCL
SA60	Ovine	Suffolk	Epididymitis	South Scotland	JCL
SA61	Ovine	Texel	Epididymitis	South Scotland	JCL
SA62	Ovine	Poll Dorset	Normal	South Scotland	JCL
SA63	Ovine	Berrichon de Cher	Normal	England	JCL
SA64[c]	Ovine	Suffolk	Normal	South Scotland	JCL
SA65[e]	Ovine	Suffolk	Normal	North Scotland	JCL
SA66[e]	Ovine	Suffolk	Normal	North Scotland	JCL
SA67	Ovine	Border Leicester	Normal	North Scotland	JCL
SA70	Ovine	Border Leicester	Epididymitis	North Scotland	JCL
SA71	Ovine	No record	Normal	No record	JCL
X16	Bovine	Not applicable	Culled	No record	Own isolate

† NCTC: National Collection of Type Cultures; JCL: Dr. J. C. Low, SACVS, Edinburgh; Own isolate: Isolated in this study (**section 2.5**).

* TAs: NCTC type strain.

c d e Isolated from samples taken at different times from the same animal.

2.3 GENERAL BACTERIOLOGICAL PROCEDURES

2.3.1 Growth media

Brain heart infusion agar (Oxoid, UK) 4.7% (w/v) supplemented with yeast extract (Oxoid) 0.5% (w/v) and defibrinated sheep blood (E and O Laboratories Ltd., Bonnybridge, UK) 5% (v/v) (BHIBYE) was used as routine solid medium for growth of these bacteria. Brain heart infusion (Oxoid) 3.7% (w/v) containing Tris (Sigma, UK) 0.1% (w/v), soluble starch (BDH, UK) 0.1% (w/v), sodium L-aspartate (Sigma) 0.5% (w/v) and thiamine monophosphate (TMP) (Sigma) 0.001% (w/v) (BHITTAS) was used as broth medium.

2.3.2 Sterilisation of culture media

All the culture media were sterilised by autoclaving at 15 lbs p.s.i (121 ^{o}C) for 15 min unless otherwise stated.

2.3.3 Storage of isolates

The isolates were received either in agar slants or as freeze dried ampoules. They were subcultured into both BHIBYE and BHITTAS and incubated as described in **section 2.3.5**. After assessing the purity (see below) they were harvested from BHIBYE plates in sterile BHITTAS containing glycerol 10% (v/v) and stored in plastic vials at -80 ^{o}C (Appuhamy *et al.*, 1997). Subsequent cultures were made from this frozen stock and subcultures of these were used for all experiments.

2.3.4 Culture purity checks

All routine agar plates and broth cultures were subjected to thorough purity checks. Agar plate cultures were checked for any unusual colonies. The broth cultures were inoculated (100 µl) onto BHIBYE plates and after incubation checked for purity of colony growth.

2.3.5 Incubation conditions

All isolates of *H. somnus, H. ovis, A. seminis* and *A. actinomycetemcomitans* in BHIBYE were incubated at 37 ^{o}C for 48 h in a candle jar. Other strains were incubated in air for 24 h at 37 ^{o}C. The experimental isolates in BHITTAS were incubated at 37 ^{o}C for 24 h in a candle jar (if they were in Universals or Bijoux) or in air (if they were in larger volumes).

2.4 IDENTITY OF TEST ISOLATES

The test isolates were received after identification in the source laboratories. To confirm their identity for this study, some of the isolates were subjected to some biochemical tests. In addition, the identity of all the isolates was determined by the API ZYM system (BioMerieux, Marcy l'Etoile, France).

2.4.1 Cultural and biochemical tests

Identity of the isolates was assessed according to the results of a panel of basic biochemical tests. These were Gram stain, motility, catalase, oxidase, indole, nitrate reduction, growth on MacConkey's agar, growth response to TMP and fermentation reactions for glucose, mannose, xylose and dulcitol. These tests were performed according to Cowan and Steel (1970), Stephens *et al.* (1983) and Hendrickson and Krenz (1991) as applicable.

2.4.2 API ZYM system

The identity of the isolates was confirmed by the API ZYM system (BioMerieux). API ZYM system is a semi-quantitative micromethod used to detect the activity of 19 enzymes (**Appendix 1.1**) produced by bacteria. The isolates were grown on BHIBYE and harvested in 0.85% (w/v) sterile saline. The cell suspension was diluted equivalent to McFarland No. 5 turbidity standard (BioMerieux). The labelled strips were inoculated with two drops of bacterial cell suspension with a Pasteur pipette into the cupules and they were incubated in a moist chamber in the dark for 4 h at 37 ^{o}C. At the end of the incubation period, all cupules were treated with one drop each of ZYM A and ZYM B (components of

the kit) and five minutes was allowed for colour development. The colour intensity was graded from 0-5 according to the API ZYM colour reaction scale.

2.5 ISOLATION OF *H. SOMNUS* FROM SLAUGHTERHOUSE SPECIMENS

Twenty two female reproductive tracts of bovine origin were obtained for the isolation of *H. somnus*, on two occasions, with 11 specimens each time. These specimens were obtained from a local slaughterhouse with kind help of Dr. H.A. Gibbs, Department of Veterinary Medicine, University of Glasgow. About five hours after slaughter, the specimens were investigated, with full aseptic procedures to expose the surfaces to be sampled. Vestibular opening, vagina, cervix and uterus were preselected as swabbing sites and the sites were swabbed in the same order each time (**Figure 2.1**). Sterile swabs were rubbed over the mucosal surface of the site and immediately inoculated onto culture plates. The inoculum was spread over the culture plate with a sterile bacteriological loop in order to obtain single colonies. Three media were used for these experiments. They were BHIBYE, MacConkey's agar (Oxoid, UK) (to detect the degree of faecal contamination) and a selective medium for *H. somnus* containing BHIBYE with horse blood 5% (v/v) and TMP 1 μg/ml and with antibiotics vancomycin (5μg/ml), neomycin (5μg/ml), sodium azide (50μg/ml), nystatin (100iu/ml) and cycloheximide (100μg/ml) (Slee and Stephens, 1985).

The inoculated plates of BHIBYE and selective medium were incubated as for *H. somnus* but the MacConkey's agar plates were incubated at 37 ^{o}C in air for 48 h. After incubation, all colonies resembling those of *H. somnus* were subcultured onto BHIBYE for confirmation of identity as above (**section 2.4**).

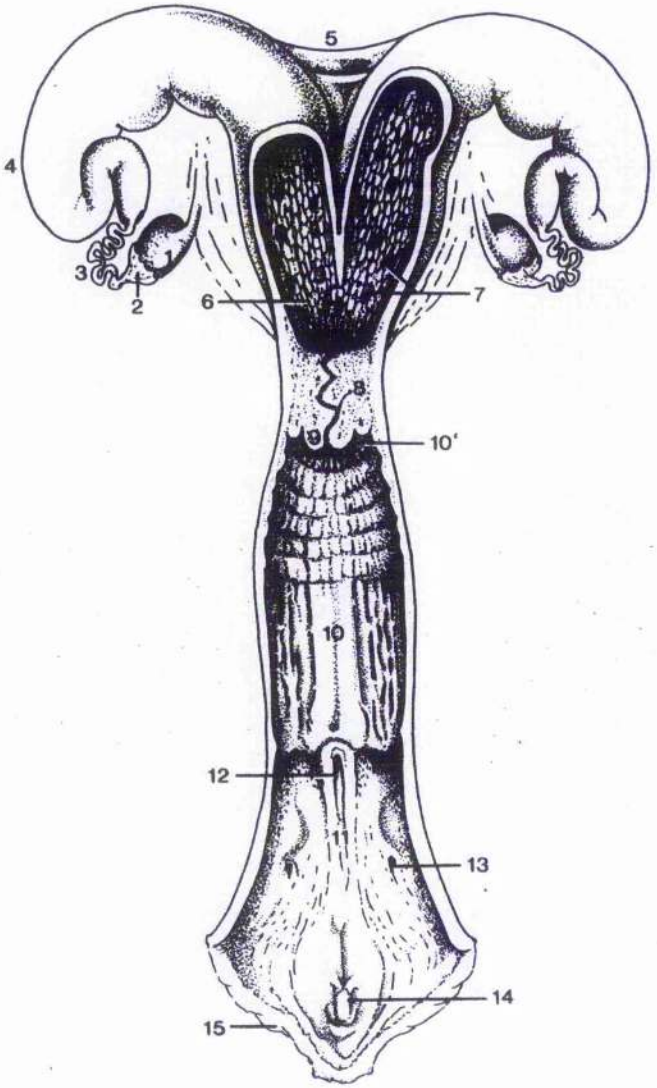

Figure 2.1 ***The reproductive tract of a cow.*** Opened dorsally. 1. ovary; 2. infundibulum; 3. uterine tube; 4. horn of uterus; 5. intercornual ligament; 6. body of uterus; 7. caruncles; 8. cervix; 9. vaginal part of cervix; 10. vagina; 10'. fornix. 11. vestibule; 12. external urethral opening; 13. opening of major vestibular gland; 14. clitoris and 15. vulva. Uterus, mid cervix, vagina and opening of the major vestibular gland were selected for attempted isolation of *H. somnus*. (This figure reproduced from Dyce *et al.* (1996) by photocopying with kind permission of W. B. Saunders, London).

2.6 CHARACTERISATION OF ISOLATES BY PCR METHODS

The main objective of this study was to identify and characterise the test isolates by different PCR techniques i.e. REP-PCR, ERIC-PCR and PCR-ribotyping.

2.6.1 Preparation of template DNA

2.6.1.1 Boiled cell extracts. Bacterial cell suspensions in sterile distilled water adjusted to a turbidity equivalent to McFarland standard No. 5 (BioMerieux, France) were prepared from bacteria grown on BHIBYE agar. A 1ml of sample in a microfuge tube was heated in a boiling water bath for 20 min. The tubes were centrifuged at 15000 x *g* in a benchtop centrifuge (Heraeus Sepatech, Germany) for 10 min and the supernate was used as the source of template DNA for PCR.

2.6.1.2 Chromosomal DNA. Chromosomal DNA was extracted by the method of Silhavy *et al.* (1984). The 24-36 h liquid cultures (10 ml) of *H. somnus* in BHITTAS were harvested by centrifugation at 3500 x *g* and the bacteria were washed twice with TE buffer (50 mM Tris, 50 mM EDTA [BDH] pH 8.0). These washed cells were suspended in 500 µl of TE buffer and frozen at -20 °C in microfuge tubes. Lysozyme (Boehringer Mannheim, UK) solution (50 µl of 10 mg/ml in 0.25 M Tris pH 8.0) was added to the frozen cells and the cells were thawed with mixing at room temperature. The thawed cells were placed on ice for 45 min and 100 µl of STEP solution (SDS 0.5% [w/v] (Sigma), 50 mM Tris (pH 7.5), 1.0 mM EDTA and proteinase K (Sigma) 1.0 mg/ml) was added. The tubes were then incubated at 60 °C for 2 h in a water bath with frequent gentle swirling. After incubation, 600 µl of phenol (liquefied washed in Tris buffer) (Fisons Scientific Equipments, Loughborough, UK) was added and mixed gently for 5 min. The contents were centrifuged at 1000 x *g* for 15 min and the aqueous phase was transferred into a clean tube. This phenol extraction was repeated once. The combined aqueous upper layers were extracted with 500 µl chloroform (Prolabo, Roger Salengro, Fonteney S/Bios), as for the phenol extraction. The DNA in the final aqueous extract was precipitated by adding 0.1 volume of sodium acetate (BDH) and 2 volumes of 100% ethanol (Fisher Scientific, Lestershire, UK). The precipitated DNA was spooled out with a hooked Pasteur pipette avoiding an excess of ethanol and transferred to a clean tube containing 500 µl of distilled water. The tubes were then placed at 4 °C overnight. The DNA suspensions were treated with 2 µl of DNAse-free RNAse (10 mg/ml) (Boehringer Mannheim) and incubated for 4 h at room temperature. Then the mixture was extracted with 500 µl chloroform twice as

above and the DNA was precipitated once again. The precipitated DNA was resuspended in 200 µl of distilled water and stored at 4 °C.

2.6.2 Primers for the PCR fingerprinting

The primers for the PCR were obtained from Genosys, Cambridge, UK and Gibco BRL, Paisley, UK in several batches. Their sequences are listed in **Table 2.4.**

2.6.3 Components of the PCR

Except where otherwise stated, the reaction mixture (25 µl) contained the final concentration of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM of each dNTP (Boehringer Mannheim, UK), 100 pM of each primer, 0.625 units of *Taq* DNA Polymerase (Life Technologies Ltd. UK) and 2.5 µl of template DNA preparation. Twenty five microliters of liquid paraffin was used to overlay each reaction. The PCR assays were performed in 1.5 ml microfuge tubes (Sarstedt Ltd., Leicester, UK).

2.6.4 Conditions for PCR

Except where stated, amplification was done in a thermocycler (Techne-PHC-2, Techne Ltd, Cambridge, UK) using 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension 72 °C for 6 min with a final extension at 72 °C for 6 min. The ramping rate was 4 °C/sec.

2.6.5 Agarose gel electrophoresis

The amplified products (7-10 µl) were mixed with 6x loading buffer (sucrose (BDH) 40% (w/v), bromophenol blue (Sigma) 0.25% (w/v)) to a final dilution of 1x and electrophoresed in 2.0% (w/v) agarose type II-A medium EEO (Sigma) in 1x Tris-borate-EDTA buffer (Tris base 89 mM, boric acid 89 mM, EDTA 2 mM (pH 8.0)) containing ethidium bromide 0.5 µg/ml (BioRad, UK) (Sambrook *et al.*, 1989) in a horizontal submarine electrophoresis apparatus (E-C Apparatus Corporation, USA). The 1Kb Ladder (GibcoBRL) was used as DNA molecular weight markers. The amplimers were visualised and photographed under UV light on a transilluminator (Model TM-40, UVP Inc., San

Table 2.4 ***Primers used in PCR typing methods***

PCR	Primer	Sequence* (5'-3')	Reference
REP-PCR	REP-IRDT	IIINCGNCGNCATCNGGC	Versalovic *et al.*, (1991)
	REP2-DT	NCGNCTTATCNGGCCTAC	
ERIC-PCR	ERIC-IR	ATGTAAGCTCCTGGGGATTCAC	Versalovic *et al.*, (1991)
	ERIC-2	AAGTAAGTGACTGGGGTGAGCG	
PCR-	GIRRN	GAAGTCGTAACAAGG	Jensen *et al.*, (1993)
Ribotyping	LIRRN	CAAGGCATCCACCGT	

* I, inosine A, adenine T, thymine C, cytosine G, guanine N, A/T/C/G

Gabriel, California, USA). The photographs were scanned with Fotolook (version 2.07.2), Agfa, UK, using a scanner Studioscan II*si*, Agfa, or documented using Ultra Violet Products Gel Documentation System-Image Store 5000, version 7.2 (Ultra Violet Products Ltd., Cambridge, UK). The electronic images were edited using Adobe Photoshop, Limited Edition 2.5.1 and the labelling of images was done with ClarisDraw version 7.5.1. Photographs were inspected visually and different band profiles were given a number or letter whenever a distinct pattern was observed.

2.6.6 Prevention of contamination of DNA and decontamination

In order to minimise the cross contamination of reagents, samples etc. and false positive results, the guidelines suggested by Kwok and Higuchi (1989) were followed. All equipment such as micropipettes (Models P2, P10, P20, P100, P200, P1000, P5000, Anachem Ltd., Luton, Beds, UK) and tips, and different areas in the laboratory were dedicated to the different stages of sample preparation, sample addition, setting up of PCR reactions, amplification and product detection. All samples, reagents and amplified products were stored in assigned boxes in separate -20 °C freezers in aliquots. The distilled water for the reaction mixture was prepared by filtration through a 0.22 µm filter (Millipore S. A., Molsheim, France) before autoclaving with single-use plastic materials and containers. All the microfuge tubes and pipette tips were autoclaved to avoid the risk of nuclease activity. The reusable equipment e.g. the tissue homogeniser, was washed with 1 M HCl between processing of samples and the micropipette barrels were treated with germicidal UV light. All the PCR experiments included negative controls without added DNA.

2.6.7 Optimisation of PCR

All PCR methods, REP-, ERIC- and PCR-ribotyping of isolates THs, SA44 and TAs representing *H. somnus*, *H. ovis* and *A. seminis* respectively, were optimised for template, deoxynucleoside triphosphate, primer and magnesium ion concentrations by a modified Taguchi method based on the use of orthogonal arrays as described by Cobb and Clarkson (1994) (**Table 2.5**). In this table, each column represents an individual reaction component and each row represents an individual reaction tube. Each component occurs at one of three predetermined levels (A, B and C) in the orthogonal array. **Table 2.6** shows the three different levels of the four components tested in terms of both its concentration in the reaction mixture and the volume of the component. Level B represents the concentration

Table 2.5 *Orthogonal array for 4 variables each at three levels*

Reaction	1	2	3	4	H_2O (µl)	Total¶
	Primer∞ (µl)	Template (µl)	$MgCl_2$ (µl)	dNTPs (µl)		
Control	**B** (2)	**B** (2.5)	**B** (1.5)	**B** (2.5)	11.875	25
1	**A** (1)	**A** (1.25)	**A** (1)	**A** (1.25)	16.875	25
2	**A** (1)	**B** (2.5)	**B** (1.5)	**B** (2.5)	13.875	25
3	**A** (1)	**C** (3.75)	**C** (2)	**C** (3.75)	10.875	25
4	**B** (2)	**A** (1.25)	**B** (1.5)	**C** (3.75)	11.875	25
5	**B** (2)	**B** (2.5)	**C** (2)	**A** (1.25)	12.625	25
6	**B** (2)	**C** (3.75)	**A** (1)	**B** (2.5)	11.125	25
7	**C** (3)	**A** (1.25)	**C** (2)	**B** (2.5)	10.625	25
8	**C** (3)	**B** (2.5)	**A** (1)	**C** (3.75)	9.125	25
9	**C** (3)	**C** (3.75)	**B** (1.5)	**A** (1.25)	9.875	25
10	**C** (3)	**C** (3.75)	**C** (2)	**C** (3.75)	6.875	25

∞ Each primer .

¶ The total volume included 2.5 µl of *Taq* buffer (Life Technologies Ltd.) and 0.125 µl of *Taq* DNA polymerase. The bold letters denote the three levels of each variable. The numbers in the brackets are the volume in µl of each component of the reaction mixture.

Table 2.6 *Concentration levels (A, B and C) for components used for the optimisation of PCR methods*

Parameter		A	B	C
1. Primer concentration	pM	50	100	150
	µl	1	2	3
2. Template DNA	µl	1.25	2.5	3.75
3. $MgCl_2$	mM	2	3	4
	µl	1	1.5	2
4. dNTPs	mM	0.1	0.2	0.3
	µl	1.25	2.5	3.75

of components in the standard reaction and this level was used as a control in the reaction. Level A is the lower level and level C is the higher level. An additional reaction (10) was included, and contained the highest levels of each component (**Table 2.5**).

The effect of annealing temperature was assessed by five different experiments for the temperatures of 40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C and 60 ^{o}C keeping all other conditions the same. In the same way the effect of extension time (1, 2, 4, 6 and 8 min) was assessed. High intensity, resolution and sharpness of amplimer bands with a low background in an agarose gel were used as the criteria for optimisation of each parameter.

2.6.8 Reproducibility of the PCR fingerprinting

The reproducibility of the amplimer band patterns was assessed not only by repeat PCR experiments with the same template sample but also with template samples derived from different cultures. The reproducibility of the patterns was also determined with different batches of *Taq* DNA polymerase and dNTPs. The reproducibility of banding pattern with different primer batches was extensively analysed, as shown in **Table 2.7**, with three primer batches from Genosys against two isolates of *H. somnus* whose patterns were different.

2.6.9 Criteria for selection of band profiles

Individual profiles were defined by the number and position of clearly visible bands. When less intense bands were visible, these were also taken into account if they were consistently present in profiles obtained on different occasions.

2.7 CHARACTERISATION OF ISOLATES BY PLASMID PROFILES

2.7.1 Plasmid DNA extraction.

Isolates were grown on BHIBYE as above, scraped off and suspended in STE buffer (100mM NaCl [BDH]; 10mM Tris-HCl [Sigma], pH 8.0; 1mM EDTA [BDH]). The cells were washed twice in STE buffer. Plasmid preparations were done with a QIAprep Spin Plasmid Kit (QIAGEN Ltd., West Sussex, UK) with minor modifications to the manufacturer's method. The procedure was based on the modified alkaline lysis method of

Table 2.7 *Detection of amplimer band pattern reproducibility with different primer batches*‡

H. somnus Isolate	Reaction	Primer batch 1		Primer batch 2		Primer batch 3
		REP-IRDT	REP-2DT	REP-IRDT	REP-2DT	REP-IRDT
THs	1	+	+			
THs	2	+			+	
THs	3		+	+		
THs	4		+			+
THs	5			+	+	
THs	6				+	+
SA01	7	+	+			
SA01	8	+			+	
SA01	9		+	+		
SA01	10		+			+
SA01	11			+	+	
SA01	12				+	+

‡ + shows the primer combination of each reaction.

Birnboim and Doly (1971) and on the adsorption of DNA onto silica in the presence of high salt. The spin columns were washed three times with PB buffer (a component of the kit) to reduce the background in the gel and plasmid DNA was eluted in 50 µl of distilled water. Ten to fifteen microlitres of plasmid DNA was analysed after electrophoresis in 0.8% (w/v) agarose type II-A (Sigma) in Tris-borate-EDTA buffer (pH 8.0) containing ethidium bromide 0.5 µg/ml.

2.7.2 Determination of antibiotic sensitivity profiles of plasmid-bearing isolates

Antibiotic sensitivity profiles of plasmid-containing and plasmidless isolates were determined by the disc diffusion method (Barry and Thornsberry, 1991) with cartridge-borne antibiotic discs. The antibiotics discs contained penicillin G (10 units), ampicillin (10 µg), augmentin (30 µg), streptomycin (25 µg), tetracycline (10 µg), cotrimoxazole (25 µg) (Mast Diagnostics, Merseyside, UK), chloramphenicol (10 µg) and enrofloxacin (Unipath Ltd., Basingstoke, UK). The 48 h cultures of test isolates on BHIBYE were suspended in sterile 0.85% (w/v) normal saline and the suspensions were standardised with a McFarland No. 3 turbidity standard (BioMerieux). A 100 µl-volume of a standardised suspension was spread onto predried BHIBYE plates and incubated at room temperature for 10 min. The antibiotic discs were then placed on the agar surface and the plates were incubated at 37 °C in a candle jar. After 48 h, the zones of inhibition were recorded with a mathematical ruler by measuring the diameter of the clear no growth area.

2.8 DEVELOPMENT OF *A. SEMINIS*-SPECIFIC PRIMERS

2.8.1 Cloning of PCR products of *A. seminis*

The aim of this procedure was to develop *A. seminis*-specific primers for the detection of *A. seminis* by PCR. The PCR-ribotyping of all *A. seminis* strains produced a similar, unique pattern. The PCR products were cloned, in order to obtain the nucleic acid sequences.

2.8.1.1 PCR-ribotyping

The PCR amplification for PCR-ribotyping was done with boiled cell extracts of *A. seminis* type strain ATCC #43626 as described previously. The primers used were GIRRN and LIRRN (**Table 2.4**). The reaction mixture (25 µl) contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM of each dNTP (Boehringer Mannheim, Lewes, UK), 100 pM of each primer, 0.625 units of *Taq* DNA polymerase (Life Technologies Ltd., Paisley, UK) and 2.5 µl of template DNA preparation. Twenty five microliters of liquid paraffin was used to overlay each reaction. Amplification was done in a thermocycler (Techne Ltd, Cambridge, UK) by 35 cycles of: denaturation at 94 °C for 30 s; annealing at 55 °C for 30 s; extension at 72 °C for 6 min. Five microlitres of amplified products were electrophoresed in 2.0% (w/v) agarose Type II-A (Sigma) in Tris-borate-EDTA buffer (pH 8.0) containing ethidium bromide (0.5 µg/ml) and the amplimers were visualised and photographed under UV light as above in order to assess the quality of the PCR products.

2.8.1.2 Purification of PCR product

Replicate samples from the above PCR-ribotyping reaction were combined and loaded (50 µl) into a 1% (w/v) agarose Type II-A (Sigma) gel with large wells and containing ethidium bromide 0.5 µg/ml and run the gel in order to obtain well resolved bands. The gel bands were excised and mashed. DNA was eluted from the agarose using GenElute agarose spin columns (Supelco, Sigma, UK). The mashed agarose was loaded into the columns and then centrifuged at 15000 x *g* for 10 min. The fluid containing DNA was eluted into microfuge tubes and subjected to ammonium acetate precipitation. The eluted DNA was mixed with 0.1 volume of 5 M ammonium acetate (BDH) and 2 volumes of ethanol 100% (v/v) pre-chilled to -20 °C and incubated at -20 °C overnight. The precipitated DNA was pelleted by centrifugation at 15000 x *g* for 15 min. The visible pellet was washed by adding 500 µl of 70% (v/v) ethanol and centrifuged at 15000 x *g* for 5 min. The pellet was air dried at 37 °C for 1 h and resuspended in 10 µl of distilled water.

2.8.1.3 'Polishing' of purified DNA products

The 'polishing' of purified DNA was done by mixing the following components in order according to the manufacturer (Stratagene Ltd, Cambridge, UK); 10 µl of purified DNA product, 1 µl of dNTP (10 mM), 1 µl of 10X cloned *Pfu* DNA polymerase buffer and 1 µl of *Pfu* DNA polymerase (2.5 U). The contents were gently mixed, centrifuged

briefly and 20 µl of liquid paraffin was used to overlay the reaction mixture. The reaction was performed by incubating the tubes at 72 °C for 45 mins.

2.8.1.4 Ligation of PCR products

The ligation was performed according to the method described by the manufacturer (Stratagene Ltd.) by mixing the following components in order; 1 µl of pCR-Script Cam SK(+) cloning vector (10 ng/µl), 1 µl of pCR-Script 10X reaction buffer, 0.5 µl of 10 mM rATP, 4 µl of PCR product, 1 µl of *Srf* I restriction enzyme (5 U/µl), 1 µl of T4 DNA ligase and 1.5 µl of distilled water to make a final volume of 10 µl. The components were gently mixed, briefly centrifuged and incubated for 1 h at room temperature. The tubes were heated for 10 min at 65 °C and then kept on ice until transformation was performed.

2.8.1.5 Transformation of cloned products

The ligated vector was transformed into supercompetent *E. coli* cells (Epicurion Coli® XL1-Blue, Stratagene Ltd.). These cells were stored at -80 °C and were thawed on ice. After gently mixing, 40 µl of the cell suspention was aliquoted into pre-chilled microfuge tubes. β-mercaptoethanol was added to each 40 µl of cell suspension to give a final concentration of 25 mM. The cell suspensions were mixed by swirling and placed on ice for 10 min, swirling gently every 2 min. Two microliters of cloned DNA was then added to each cell suspension, the mixture swirled gently and placed on ice for 30 min. These cell suspensions were subjected to a heat pulse at 42 °C in a water bath for 45 s and then this transformation mixture was placed on ice for 2 min. Four hundred and fifty microlitres of preheated (42 °C) SOC medium was added. SOC medium consisted of 20 g of tryptone [Oxoid, UK], 5 g of yeast extract [Oxoid, UK], 0.5 g of NaCl [Fisher Scientific UK Ltd., Leicestershire, UK] dissolved in 970 ml of distilled water and sterilised by autoclaving and then 10 ml of 1 M $MgCl_2$ [Fisher, UK], 10 ml of 1 M $MgSO_4$ [Fisher, UK] and 10 ml of glucose 20% (w/v)[BDH], all filter sterilised (0.45 µm), were added. The mixture was incubated at 37 °C for 1 h with shaking at 200 rpm. After incubation the cell mixtures were plated onto Luria-Bertani (LB)-chloramphenicol agar (per litre; 10 g of tryptone [Oxoid, UK], 10 g of yeast extract [Oxoid, UK], 0.5 g of NaCl [Fisher, UK], 20 g agar [Oxoid, UK], 30 mg of filter sterilised chloramphenicol [Sigma] dissolved in 100% ethanol [Fisher], 1.6 ml of 5% X-gal [Sigma] and 5 ml of 100 mM IPTG [Sigma]) and plates were incubated at 37 °C overnight. After incubation, the LB plates were stored at 4 °C for 1 h to enhance the blue colour of colonies which contained only ligated vector

molecules. White and pale blue colonies were subcultured on to LB-chloramphenicol agar without X-gal and IPTG and incubated at 37 °C overnight.

2.8.1.6 Screening of colonies for the insert

The screening was done by two methods. **PCR method:** All the white colonies and pale blue colonies were subcultured onto separate LB agar plates containing chloramphenicol. A single blue colony from each transformation was subcultured to use as a negative control. From overnight culture plates 2-3 colonies were suspended in 100 µl of distilled water and the template DNA was prepared by boiling (**section 2.6.1.1**). PCR was performed for these templates with ribotyping LIRRN and GIRRN primers (Jensen *et al.*, 1993) under the standard conditions (**section 2.6.4**). The products were analysed as above (**section 2.6.5**).

Restriction digestion method: The plasmid DNA from putative transformants was extracted using QIAprep spin column (mini) kits (QIAgen). The quality of plasmid DNA extracted was assessed by 0.8% (w/v) agarose gel electrophoresis. The plasmid DNA preparations were double digested with 1 µl of each of restriction enzymes *Sac*I (Stratagene Ltd, Cambridge, UK) and *Eco*R I (Life Technologies, Paisley, UK). The 20 µl reaction mixture contained 5 µl of plasmid DNA, 1 µl of each restriction enzyme, 5 µl of universal buffer (1 M potassium acetate, 250 mM Tris-acetate (pH 7.6), 100 mM magnesium acetate, 5 mM β-mercaptoethanol, bovine serum albumin 100 µg/ml) (Stratagene Ltd, Cambridge, UK) and 8 µl of distilled water. The reaction conditions were 37 °C in a heat block for 1 h. The sizes of the insert were assessed after running the digest in 2% (w/v) agarose gel with ethidium bromide (0.5 µg/ml) and visualisation under UV light (UVP Inc.).

2.8.2 Sequencing of PCR-ribotyping products of *A. seminis*

2.8.2.1 Cycle sequencing by PCR

The double-stranded plasmid DNA template for sequencing was prepared using QIAprep (mini) spin columns (QIAgen). The concentration of DNA was measured by a spectrophotometric method (Sambrook *et al.*, 1989). The sequencing was performed by cycle sequencing and dye terminator method (Lee *et al.*, 1992). Sequencing was performed from both ends of the insert. The primers were: M13-21 5' TGTAAAACGACGGCCAGT 3' for the plus strand and T3 primer 5' ATTAACCCTCACTAAAGGGA 3' for the minus

strand. These primers were kindly provided by Dr. Veer Math of the Molecular Biology Support Unit, Institute of Biomedical and Life Sciences (IBLS), University of Glasgow. The sample for PCR contained 8 µl of Terminator Ready Reaction Mix (Perkin-Elmer, Warrington, UK), 3.2 pmole of primer, 400 ng of double-stranded template DNA and the volume was adjusted with distilled water to 20 µl. One drop of light mineral oil was used to overlay the reaction mixture. The tubes containing reaction mixture were placed in a thermal cycler (DNA Thermal Cycler Model 480, Perkin-Elmer). The tubes were subjected to 25 thermal cycles. Each cycle consists of: rapid thermal ramp to 96 ^{o}C, 96 ^{o}C for 30 s, rapid thermal ramp to 50 ^{o}C, 50 ^{o}C for 15 s, rapid thermal ramp to 60 ^{o}C, 60 ^{o}C for 4 min. At the end of 25 cycles the temperature was rapidly ramped to 4 ^{o}C and held until ethanol precipitation. The sequencing was repeated to determine the accuracy by replacing the T3 primer with Reverse primer 5' TTCACACAGGAAACAG 3' (Amersham International plc, Slough, UK) for the minus strand.

2.8.2.2 Purifying the extension products

The entire 20 µl of reaction mixture containing the extension products was transferred to a 0.5 ml microfuge tube containing 2 µl of 2 M sodium acetate (pH 4.6) and 50 µl of 95% (v/v) ethanol. The tubes were tapped to mix, centrifuged briefly, and incubated at -80 ^{o}C for 10 min. The tubes were centrifuged at 15000 x *g* for 15 min. The ethanol was carefully aspirated. The pellet was washed with 250 µl of 70% (v/v) ethanol without centrifuging and dried in a vacuum centrifuge for 8 min.

2.8.2.3 Running of the sequencing gel

The dried pellet was resuspended by brief vortexing in 4 µl of loading solution for running of samples in the gel. The loading solution (Perkin Elmer) contained deionised formamide: 50 mM EDTA in the ratio 5:1. The samples were then heated at 90 ^{o}C for 2 min and placed on ice until ready to load. The samples were run on a standard 4.5% (w/v) acrylamide (19:1 acrylamide: bis acrylamide) gel in an Applied Biosystems 373 DNA Sequencer, Perkin Elmer.

2.8.2.4 Analysis and comparison of sequences

The sequences generated were analysed and aligned using the computer software Sequence Navigator® version 1.0 (Applied Biosystems). The accuracy of the sequences was determined by repeat experiment and by comparing the electropherograms of each base generated by the sequencing machine. The sequences were then compared with the database BLAST search (Altschul *et al.*, 1990) using Netscape Navigator™ version 2.02 (Netscape Communications Corporation, USA). The regions of the RNA operons were defined by comparing the sequences with those in the Genbank database and by use of the guidelines suggested by Gurtler and Stanisich (1996). The spacer regions of rRNA operons of *A. seminis* were aligned with fig. 2 of Gurtler and Stanisich (1996) (The electronic form of fig 2 was kindly provided by Dr. V. Gurtler via electronic mail).

2.9 DESIGN OF *A. SEMINIS*-SPECIFIC PRIMERS

2.9.1 Primers for specific PCR

The primers were designed after comparison of the sequences with the database of rRNA operons and spacer regions in order to obtain a PCR product of 300-500 bp amplifiable only in *A. seminis*. The sequence for reverse primer SRJAS1 (5' CTTATCTTTCTTAAGCCCTGAC 3') was selected from an area that had no match with the sequences in the database. The forward primers SRJAS2 (5' AAGAAAAAGACGAAGAGACATT 3') and SRJAS3 (5' AATTGAGTGAGAGTGAAAGC 3') were selected from regions where no homology was found with any known bacterial sequences. The primers were purchased from Life Technologies Ltd.

2.9.2 Optimisation of specific PCR

The components of the reaction mixture were optimised for template, dNTPs, primer and magnesium ion concentrations by a modified Taguchi method based on the use of orthogonal arrays as described by Cobb and Clarkson (1994). Annealing temperature was raised from 50 °C to 60 °C in order to increase the specificity of the assay. Again, high intensity, resolution and sharpness of amplimer bands with a low background in an agarose gel were used as the criteria for optimisation.

2.9.3 Specificity of primers

The specificity of the assay was assessed by testing the primers SRJAS1 and SRJAS2 with all bacterial isolates listed in **section 2.1**. The bacterial cell suspensions in sterile distilled water, with a turbidity equivalent to McFarland standard No. 5 (BioMerieux), were prepared from bacteria grown on BHIBYE agar. Samples of 1 ml were heated in a boiling water bath for 20 min. The tubes were then centrifuged at 15000 x *g* for 10 min and the supernates was used as the source of template DNA for PCR.

2.9.4 Sensitivity of primers

The sensitivity of the PCR assay was determined with ram semen samples deliberately contaminated by adding known number of *A. seminis* colony forming units (CFU) and also with ram semen naturally infected with *A. seminis* to different degrees. An *A. seminis* isolate (SA63) was grown on BHIBYE (**section 2.3.5**) and a bacterial cell suspension was made in sterile distilled water to a turbidity equivalent to McFarland standard No. 5 (BioMerieux). Ten-fold serial dilutions were made in distilled water up to dilution 10. A 100 µl sample of each dilution were spread in to BHIBYE in duplicate to determine the CFU in each dilution and plates were incubated as described earlier. A 100 µl-volume of each bacterial dilution was used for preparation of template DNA for PCR by boiling. For some samples, the sensitivity was tested by incorporation of Chelex® 100 (BioRad, Herts, UK) at a final concentration of 5% (w/v) (de Lamballerie *et al.*, 1992). The sensitivity of the assay was also monitored with ram semen without and with storage solution. The sensitivity was tested after treating the samples with proteinase K lysis buffer (10 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 5 mM EDTA and 400 µg of proteinase K [SigmaJ) (Domeika *et al.*, 1994).

2.9.5 Detection of *A. seminis* in naturally-contaminated semen

Naturally-infected ram semen specimens from which *A. seminis* had previously been isolated and identified by biochemical phenotyping and by API ZYM were also tested. Six semen samples were assessed for the level of *A. seminis* contamination initially by plate counts. They were: two heavily contaminated specimens (both had between 7 and 8 x 10^7 CFU/ml) and a low-contaminated specimen (150 CFU/ml) without storage solution and three semen samples in storage solution (containing *A. seminis* at 40-180 CFU/ml). These semen samples were diluted 1 in 10 and 1 in 100 and subjected to PCR with primers

SRJAS1 and SRJAS2 after treatment with proteinase K and boiling in the presence of 5% (w/v) Chelex 100.

2.9.6 Detection of *A. seminis* in tissues

Four tissue samples from the reproductive tract of a ram with epididymitis and orchitis were kindly provided by Dr. J. C. Low. The testes were swollen and reddened and the semen ejaculate contained *A. seminis*. The tissue samples showing gross lesions were from the left testis, left caput epididymis, left cauda epididymis and left prostate gland and were collected aseptically from the euthanised ram and cultured for *A. seminis* by Dr. J. C. Low. Only the sample from the caput epididymis showed the presence of *A. seminis* at a low level, but the colony count was not recorded. The tissue samples were suspended in approximately 10 volumes of filter sterilised (0.22 μm) distilled water and homogenised with tissue homogeniser (Silverson Machines Ltd.). The homogenised samples were centrifuged at 500 x *g* for 1 min. The supernates were transferred to new microfuge tubes and serial 10-fold dilutions up to 10000-fold were made. Each dilution of each sample was treated with proteinase K as described previously and boiled with 5% (w/v) Chelex 100. The template DNAs for PCR were then prepared as in **section 2.6.1.1.**

2.10 NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The Genbank accession numbers of *A. seminis rrnA* and *rrnB* are AF013275 and AF013276 respectively.

3. RESULTS

3.1 IDENTITY OF ISOLATES

3.1.1 Cultural and biochemical properties

3.1.1.1 *H. somnus*

For all of the isolates described as *H. somnus*, the colonies were convex, entire, moist, glistening and yellow or grey-yellow colour. The size of the colonies after 48 h of incubation on BHIBYE in a candle jar, varied between isolates from 1-2 mm in diameter. They grew in air but the colonies were much smaller than after growth in the candle jar. There was little improvement of colony size in air or in the candle jar after prolonged incubation. The isolates were Gram-negative, pleomorphic and non-motile. All showed positive results for oxidase, indole production and nitrate reduction tests and most of the isolates showed enhanced growth response for TMP when present in the medium (26/29) (**Table 3.1**). They were catalase negative and did not grow on MacConkey agar. All fermented glucose (acid only, no gas) and did not ferment dulcitol but showed variable results (acid only, no gas) for mannose and xylose (**Table 3.1**). None of these isolates produced gas in fermentation tests. The *H. somnus* isolates obtained from slaughterhouse specimens (**section 2.5**) had similar properties as other *H. somnus* isolates (**Table 3.1**).

3.1.1.2 *H. ovis*

The cultural and biochemical characteristics of the isolates provided as *H. ovis* were the same as those of *H. somnus* except that the colonies were less yellow than those of *H. somnus*. None of the isolates grew on MacConkey's agar. The fermentation reactions were the same as for *H. somnus*, with variable results (acid only, no gas) for mannose and xylose but a higher proportion of *H. ovis* isolates produced acid from mannose than *H. somnus* isolates (**Table 3.1**).

3.1.1.3 *A. seminis*

The colonies of all the isolates described as *A. seminis* were tiny (<1 mm) after incubation for 24 h and greyish-white, shiny, circular, low convex and about 2 mm in diameter after 48 h. They became 3-4 mm or even larger after incubation for >96 h at 37 °C in a candle jar. Microscopically, the bacteria were Gram-negative, pleomorphic and non-motile. The umbonate shape of the colonies was apparent after incubation for 48 h. All of

Table 3.1 ***Biochemical and fermentation properties of H. somnus and H. ovis isolates***

H. somnus Isolate	Growth response to TMP	Mannose	Xylose
THs	+	--	+
SA01	--	(+)	+
SA02	+	--	+
SA03	+	--	+
SA04	+	--	+
SA05	+	--	+
SA06	--	--	+
SA07	+	--	+
SA11	+	--	+
SA13	+	--	+
SA14	+	--	+
SA15	+	--	+
SA17	+	--	+
SA19	+	--	+
SA20	--	(+)	--
SA21	+	--	(+)
SA22	+	--	(+)
SA23	+	--	(+)
SA48	+	+	+
SA49	+	--	+
SA50	+	+	+
SA51	+	--	+
SA52	+	--	+
SA68	+	--	+
V3	+	--	+
V8	+	(+)	+
X1	+	--	+
X4	+	--	+

H. ovis Isolate	Growth response to TMP	Mannose	Xylose
SA08	+	+	+
SA16	+	+	+
SA24	+	--	+
SA26	+	+	+
SA27	--	+	+
SA28	+	+	--
SA29	--	--	+
SA44	+	+	+
SA45	+	--	+
SA46	+	--	+
SA53	+	+	+
SA54	+	+	+
SA55	+	+	+
SA56	--	+	+
SA57	+	+	+
SA58	+	+	+
SA69	+	+	+
SA72	ND	ND	ND
SA73	ND	ND	ND

All *H. somnus* and *H. ovis* isolates were Gram-negative and non-motile. All these isolates were positive for oxidase, nitrate reduction, indole tests and produced acid but no gas with glucose. The isolates were negative for catalase, growth on MacConkey's agar and had no reaction with dulcitol. Variable results were given for growth response to TMP, mannose and xylose. +, positive (acid only, no gas); (+), weak positive; --, no reaction and ND, not done.

the *A. seminis* isolates were catalase, oxidase and nitrate positive, negative for indole production and growth on MacConkey's agar. None of the *A. seminis* isolates tested produced a reaction with mannose and dulcitol fermentation reactions. Only three isolates produced acid from glucose while 11 isolates produced acid (no gas) from xylose (**Table 3.2**). The bovine isolate (X16) showed similar properties to other ovine *A. seminis* isolates (**Table 3.2**).

3.1.2 API ZYM assay

The different enzymic tests and the colour grades obtained in the API ZYM assay for individual isolates of the three species, *H. somnus, H. ovis* and *A. seminis* are detailed in the **Appendix 6.1.2, 6.1.3** and **6.1.4**. The patterns of variation among these three species are summarised in **Table 3.3**. The *P. haemolytica* isolate and α-chymotrypsin control tests gave the expected reactions (**Appendix 6.1.2**).

3.1.2.1 *H. somnus*

The *H. somnus* isolates were consistantly positive in only three enzymic tests (leucine arylamidase (6), acid phosphatase (11) and β-glucuronidase (15)). In another five tests, namely alkaline phosphatase (2), esterase (C 4) (3), esterase lipase (C 8) (4), naphthol-AS-BI-phosphohydrolase (12) and α-fucosidase (20), which showed strong reaction when positive, the isolates gave variable reactions (**Table 3.3**).

3.1.2.2 *H. ovis*

All 19 of the *H. ovis* isolates showed consistently positive reactions for leucine arylamidase (6), acid phosphatase (11) and β-glucuronidase (15). The tests for alkaline phosphatase (2), esterase (C 4) (3), esterase lipase (C 8) (4), naphthol-AS-BI-phosphohydrolase (12) and α-fucosidase (20) also yielded variable results for these isolates (**Table 3.3**).

Table 3.2 ***Biochemical and fermentation properties of A. seminis isolates***

A. seminis isolate	Glucose	Mannose	Xylose	Dulcitol
TAs	--	--	--	--
SA25	+	--	+	--
SA30	--	--	(+)	--
SA31	--	--	(+)	--
SA32	+	--	+	--
SA33	--	--	+	--
SA34	--	--	--	--
SA35	--	--	+	--
SA36	--	--	+	--
SA37	--	--	(+)	--
SA38	--	--	+	--
SA39	--	--	+	--
SA43	--	--	--	--
SA60	ND	ND	ND	ND
SA61	ND	ND	ND	ND
SA62	ND	ND	ND	ND
SA63	ND	ND	ND	ND
SA64	ND	ND	ND	ND
SA65	ND	ND	ND	ND
SA66	ND	ND	ND	ND
SA67	ND	ND	ND	ND
SA70	ND	ND	ND	ND
SA71	ND	ND	ND	ND
X16	+	--	+	--

All *A. seminis* isolates were Gram-negative and non-motile. All the isolates tested were positive for catalase, oxidase, nitrate reduction tests and negative for indole, growth on MacConkey's agar and did not ferment mannose and dulcitol. Variable results were given by the isolates tested for fermentation reactions of glucose and xylose. +, positive (acid only, no gas); (+), weak positive; --, no reaction and ND, not done.

Table 3.3 ***Identification of isolates by API ZYM and comparison of three species***

Isolates	Number of isolates	Activity of enzyme on API ZYM substrates (Test No.)							
H. somnus **isolates**	**29**	**2**	**3**	**4**	**6**	**11**	**12**	**15**	**20**
THs, SA21, SA22, SA23, SA50	5				+	+		+	
SA17, SA19, SA20, V8, X4	5	+	+	+	+	+		+	
SA01, SA03, SA04, SA05, SA11	5	+	+	+	+	+	+	+	
SA49, SA51, SA52, V3	4	+			+	+		+	
SA12, SA13	2	+		+	+	+	+	+	
SA14, SA15	2	+	+	+	+	+	+	+	+
SA02	1	+			+	+	+	+	
SA06	1	+		+	+	+	+	+	+
SA07	1	+	+	+	+	+		+	
SA48	1				+	+		+	+
SA68	1	+		+	+	+		+	
X1	1				+	+	+	+	+

H. ovis **isolates**	**19**	**2**	**3**	**4**	**6**	**11**	**12**	**15**	**20**
SA24, SA26, SA27, SA29, SA44, SA45, SA46, SA53, SA55, SA57, SA58, SA69	12				+	+		+	
SA28, SA54, SA72	3	+			+	+		+	
SA08, SA16	2	+	+	+	+	+	+	+	
SA56	1	+		+	+	+		+	
SA73	1	+			+	+	+	+	

A. seminis **isolates**	**24**	**2**	**3**	**4**	**6**	**11**	**12**	**15**	**20**
TAs, SA25, SA30, SA31, SA32, SA33, SA34, SA36, SA37, SA38, SA39, SA43, SA60, SA61, SA62, SA63, SA64, SA65, SA66, SA67, SA70, SA71	22	+			+	+		+	
SA35	1	+		+	+	+		+	
X16	1				+	+		+	

3.1.2.3 *A. seminis*

All 24 *A. seminis* isolates, including the bovine isolate (X16) showed strong positive reactions for leucine arylamidase (6), acid phosphatase (11) and β-glucuronidase (15). For the reaction of alkaline phosphatase, variable intensities were observed and the bovine isolate X16 was negative for this reaction. Only one isolate (SA35) showed a weak positive reaction for esterase lipase. All other tests were negative (**Table 3.3**).

3.1.3 Comparison of the three species by API ZYM

The isolates of all 3 species showed positive reactions in tests for leucine arylamidase (6), acid phosphatase (11) and β-glucuronidase (15) and these showed stronger colour reactions than the other positive tests in the assay. Among the three species, *A. seminis* isolates in general showed the strongest colour reactions for these three tests and for the other positive reactions. *H. somnus* isolates showed the highest variability in terms of intensity of positive reactions among the 19 tests and in the number of positive tests among the isolates. Only some of *H. somnus* isolates showed a positive reaction for α-fucosidase (20) but none of the *H. ovis* or *A. seminis* isolates were positive (**Table 3.3**).

3.2 ISOLATION OF *H. SOMNUS* AND *A. SEMINIS* FROM SLAUGHTERHOUSE MATERIALS

Four of 22 reproductive tracts of cows yielded *H. somnus*. The isolate V3 was from the vestibular opening of the tract and that particular uterus contained a mucopurulent discharge. Similar isolates were also recovered from the cervix and the uterus of the same animal. The swabs from these sites yielded other bacteria as well. The V8 isolate was from the cervix of another cow and was a pure culture. The vagina of this tract was full of mucopurulent discharge. Similar pure cultures were obtained from the vagina and the uterus as well but the vestibular opening yielded a pure culture of another unidentified bacterium. The X1 isolate was obtained from the cervix of an apparently healthy tract and similar colonies were present in cultures from the vestibular opening and vagina but not from the uterus. The isolate X4 was present in the swab from the vagina and similar colonies were present in samples from the cervix and vestibular opening but not from the uterus. The isolation of these *H. somnus* strains was made from the selective medium as it reduced the number and types of contaminants. Similar colonies however were observed from corresponding samples inoculated on some of the non-selective plates containing

BHIBYE agar. The identity of these isolates was similar to other *H. somnus* isolates on the basis of cultural and biochemical properties. The isolate X16 subsequently identified as *A. seminis* was isolated on a plate containing BHIBYE agar, from the vestibular opening of a different tract which showed vaginitis. The presence of this strain in the other sites of the same tract was not established as those plates were overgrown by other bacteria.

3.3 CHARACTERISATION OF ISOLATES BY PCR

3.3.1 Optimisation of PCR

The optimised level of the components of the reaction mixture, i.e. giving the best resolution and highest intensity of the amplimer bands, are summarised in **Table 3.4**. For *H. somnus* type strain THs, the optimised levels were 0.3 mM dNTPs, 3 mM $MgCl_2$, 100 pM of each primer and 1.25 µl of template preparation, that is reaction 4 in **Table 2.5**. These levels were the same for all three PCR methods and are shown in lanes 4 for REP-PCR, ERIC-PCR and PCR-ribotyping in **Figure 3.1**. The highest resolution and intensity of the amplimer bands for *H. ovis* strain SA44 were obtained with 0.3 mM dNTPs, 4 mM $MgCl_2$, 50 pM of each primer and 3.75 µl of template preparation as in reaction 3 of **Table 2.5**. These levels were the same for all three PCR methods and as shown in lanes 3 of **Figure 3.2**. For *A. seminis* the reaction "control" (**Table 2.5**), lanes **C** for REP-PCR, ERIC-PCR and PCR-ribotyping gave the best resolution and intensity (**Figure 3.3**). The optimised annealing temperature for *H. somnus* and *H. ovis* for all three methods was 50 °C (lanes 3, **Figure 3.4**) but for *A. seminis* it was 55 °C for all three methods (lanes 4, **Figure 3.4**). The extension time of 6 min was found to be the most appropriate for all three species and all three methods and gave superior band patterns to those obtained with extension times of 1, 2, 4 and 8 min (data not shown).

3.3.2 Fingerprinting of *H. somnus*

3.3.2.1 *REP-PCR*

With the REP-PCR method, the profiles of *H. somnus* revealed amplified bands ranging from <0.28-1.4 kb with various intensities (**Figure 3.5**), of which 13 were used to determine fingerprints. The 29 isolates showed 11 distinct patterns or fingerprints, each of which was assigned a number (**Table 3.5**). Group **1** included the type strain and 10 other isolates with essentially identical patterns and this was the largest group. Groups **2, 3** and **4** each contained three isolates. Groups 5 and 6 each comprised two strains with

Table 3.4 ***Optimised reaction mixture for the three species of bacteria***

Component	*H. somnus*	*H. ovis*	*A. seminis*
Taq buffer	2.5 µl	2.5 µl	2.5 µl
dNTPs	3.75 µl	3.75 µl	2.5 µl
$MgCl_2$	1.5 µl	2.0 µl	1.5 µl
Taq DNA polymerase	0.125 µl	0.125 µl	0.125 µl
[a]Primer 1	2.0 µl	1.0 µl	2.0 µl
[b]Primer 2	2.0 µl	1.0 µl	2.0 µl
Template preparation	1.25 µl	3.75 µl	2.5 µl
Distilled water	11.875 µl	10.875 µl	11.875 µl
Total	25.0 µl	25.0 µl	25.0 µl
Liquid paraffin	25.0 µl	25.0 µl	25.0 µl

[a]Primer 1: REP-IRDT, ERIC-IR and GIRRN

[b]Primer 2: REP2-DT, ERIC-2 and LIRRN

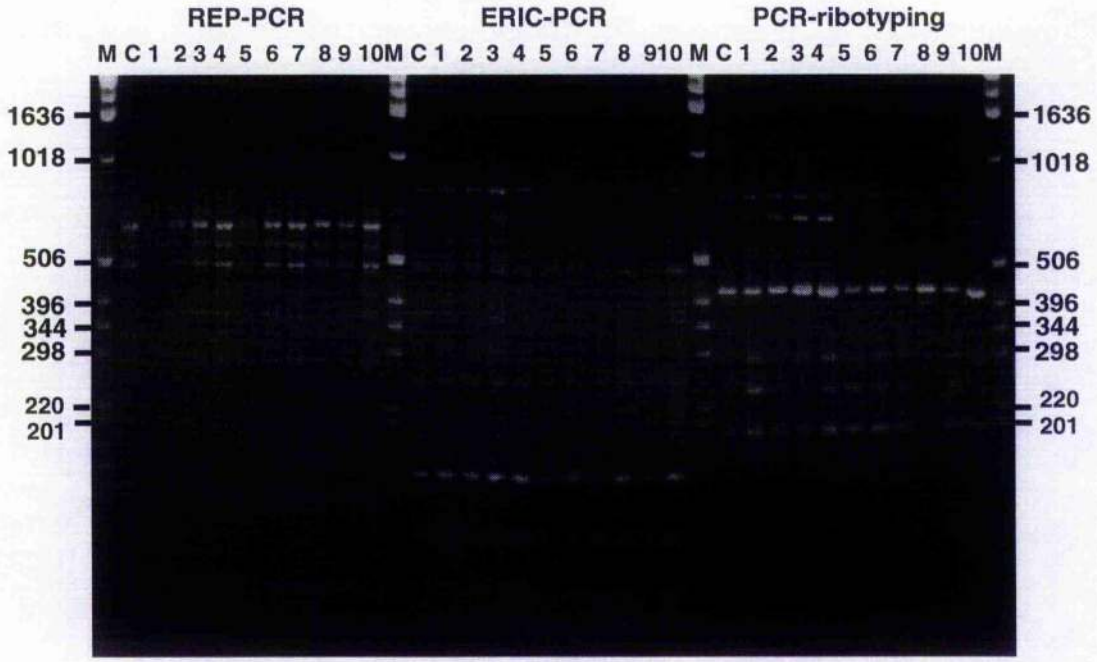

Figure 3.1 ***Optimisation of PCR mixture for H. somnus***. Lanes M: 1 Kb DNA Ladder. Lanes C and 1-10 in each group, REP-PCR, ERIC-PCR and PCR-ribotyping, correspond to the reactions listed in **Table 2.5** respectively.

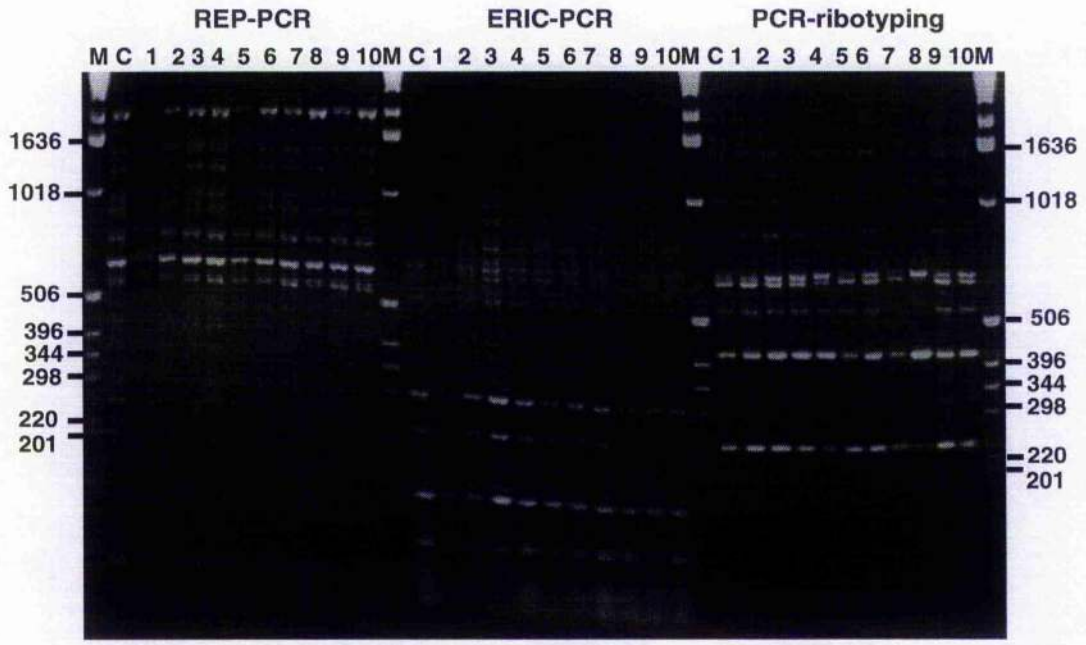

Figure 3.2 ***Optimisation of PCR mixture for H. ovis.*** Lanes M: 1 Kb DNA Ladder. Lanes C and 1-10 in each group, REP-PCR, ERIC-PCR and PCR-ribotyping, correspond to the reactions listed in **Table 2.5** respectively.

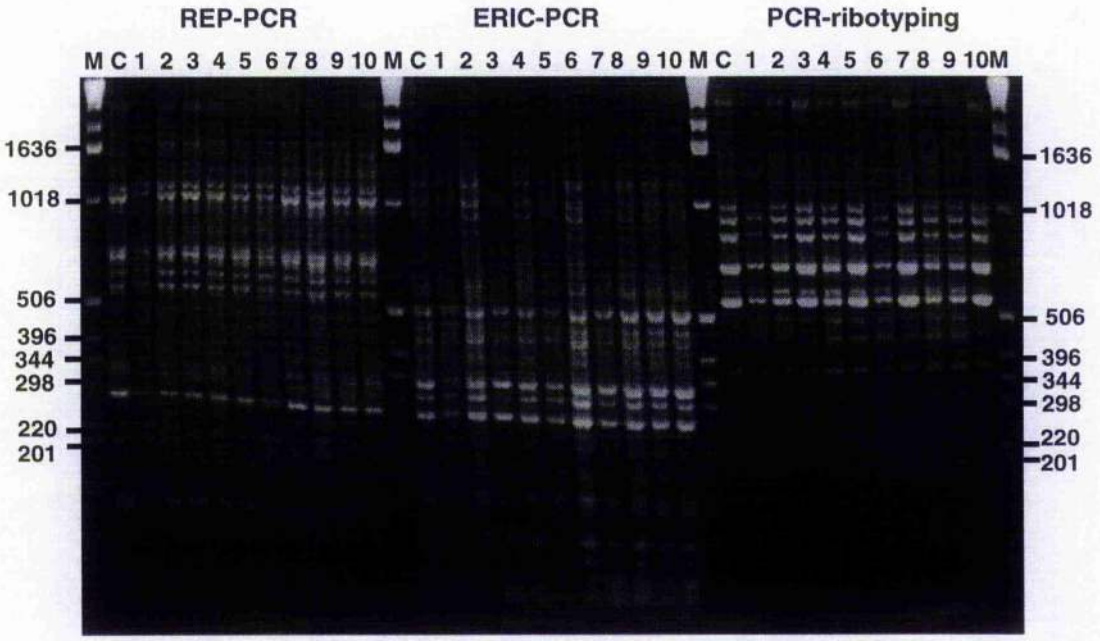

Figure 3.3 ***Optimisation of PCR mixture for A. seminis.*** Lanes M: 1 Kb DNA Ladder. Lanes C and 1-10 in each group, REP-PCR, ERIC-PCR and PCR-ribotyping, correspond to the reactions listed in **Table 2.5** respectively.

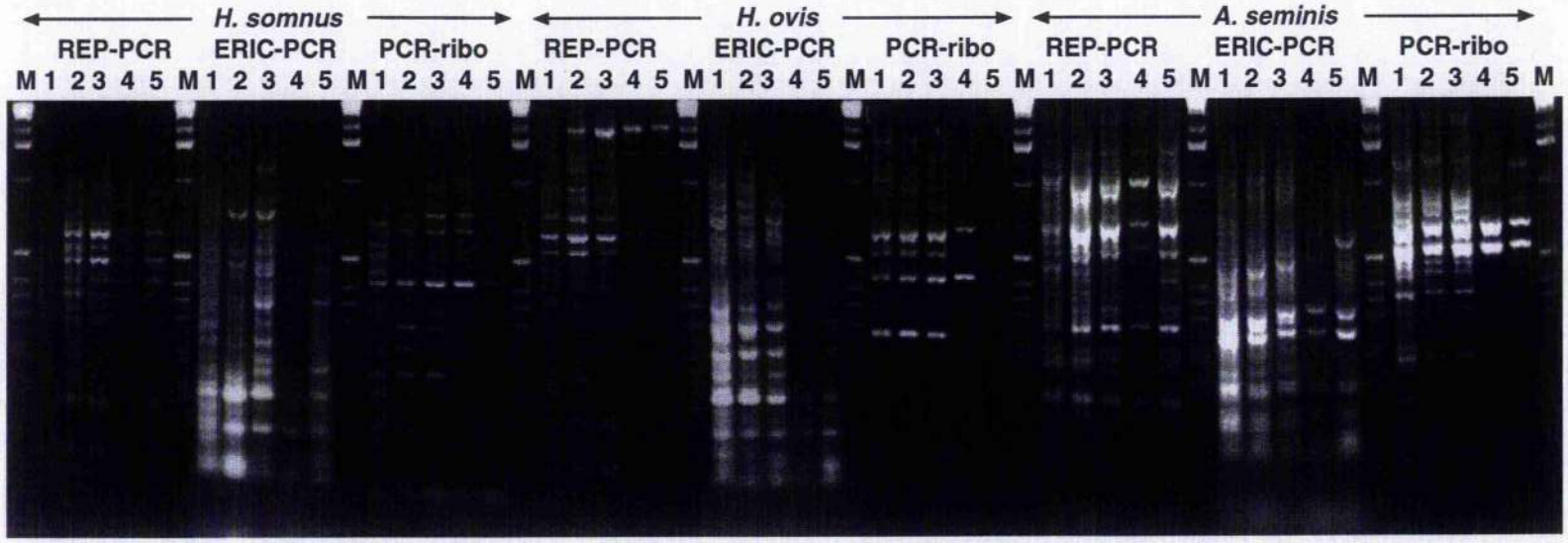

Figure 3.4 ***Optimisation of annealing temperature for PCR with the three species.*** Lanes M: 1 Kb DNA ladder. Lanes 1-5: different experiments to determine the effect of different annealing temparatures, 40 °C, 45 °C, 50 °C, 55 °C and 60 °C, respectively. Experiment 4 did not perform well and so the band intensity was not good. Reactions were run with the optimised reaction mixtures (**Table 3.4**).

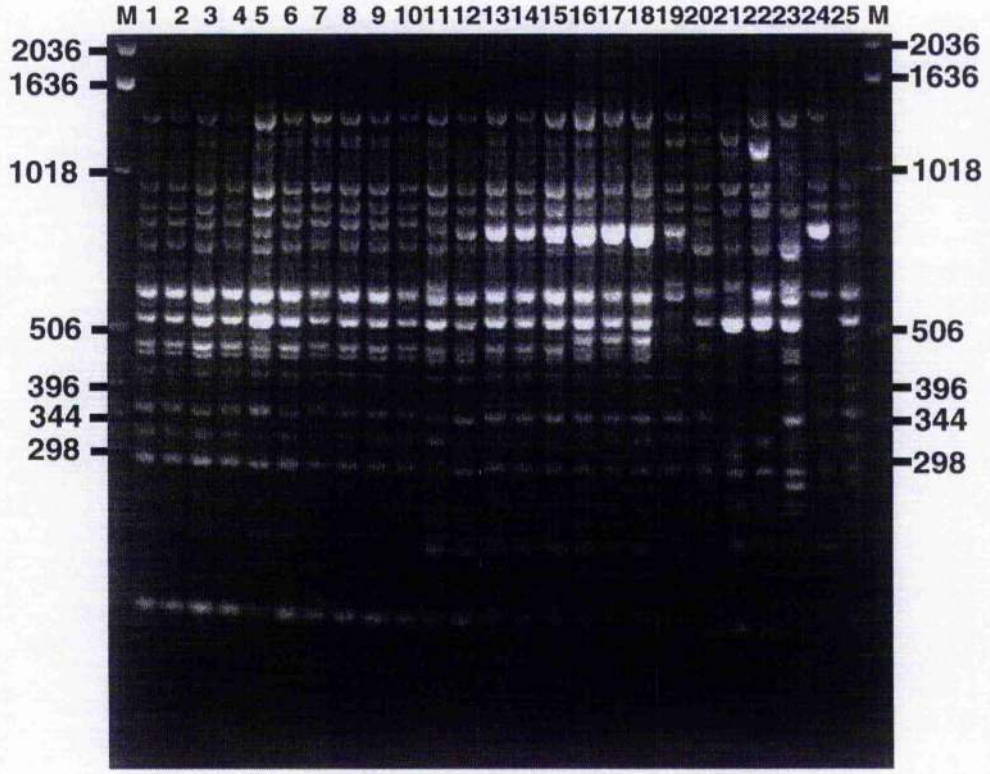

Figure 3.5 ***Fingerprints obtained by REP-PCR for H. somnus isolates.*** Lanes M; 1 kb ladder. Lanes 1-20 and 23-25: *H. somnus* isolates; THs (type strain), SA05, SA06, SA07, SA12, SA14, SA15, SA17, SA19, SA20, V3, X1, SA21, SA22, SA23, SA01, SA02, SA03, SA13, SA11, SA04, V8 and X4 respectively. Lanes 21 and 22: *H. ovis* isolates SA08 and SA16 respectively. Isolates SA48, SA49, SA50, SA51, SA52, SA68 are not included in this figure. The profiles have been arranged so that, in general, isolates of a similar type are grouped together. Lanes 1-4 and 6-10 (type 1), lane 5 (type 6), lane 11 (type 10), lane 12 (type 9), lanes 13-15 (type 3), lanes 16-18 (type 2), lane 19 (type 8), lane 20 (type 7), lane 23 (type 4), lane 24 (type 11) and lane 25 (type 5) (see **Table 3.5**)

Table 3.5 *Types of H. somnus isolates by three different typing methods*

REP types (11 types)	Isolates	No. of isolates
1	THs, SA05, SA06, SA07, SA14, SA15, SA17, SA19, SA20, SA50, SA51	11
2	SA01, SA02, SA03	3
3	SA21, SA22, SA23	3
4	SA04, SA52, SA68	3
5	SA49, X4	2
6	SA12, SA48	2
7	SA11	1
8	SA13	1
9	X1	1
10	V3	1
11	V8	1

ERIC types (16 types)	Isolates	No. of isolates
A	THs, SA05, SA06, SA14, SA15, SA19, SA20, SA48, SA50, SA51	10
B	SA01, SA02, SA03,	3
C	SA21, SA22, SA23,	3
D	SA07	1
E	SA13	1
F	SA17	1
G	SA04	1
H	V3	1
I	SA52	1
J	V8	1
K	X4	1
L	SA49	1
M	SA12	1
N	X1	1
O	SA11	1
P	SA68	1

Table 3.5 continued...

Table 3.5 continued...

Ribotypes (8 types)	Isolates	No. of isolates
a	THs, SA05, SA06, SA14, SA15, SA17, SA19, SA20, SA48, SA50, SA51	11
b	SA01, SA02, SA03, SA04, SA21, SA22, SA23, SA68	8
c	SA12, SA13, V3	3
d	SA07, SA11, X4	3
e	SA52	1
f	X1	1
g	V8	1
h	SA49	1

similar patterns. The remainder of the *H. somnus* isolates each produced unique patterns. There was close similarity of fingerprints among groups **1, 2, 3** and **4** showing common markers but other groups did not share those markers (**Figure 3.5**).

3.3.2.2 *ERIC-PCR*

ERIC-PCR produced 16 distinguishable patterns, each of which was assigned a letter (upper case), for the 29 *H. somnus* isolates (**Table 3.5**). The fragment sizes ranged from <0.1-2.2 kb with various band intensities (**Figure 3.6**) and 13 of these were used to assign fingerprints. This method produced a higher degree of discrimination between these isolates, but the complex banding patterns were more difficult to interpret. Again the type strain fell into the largest group (group **A**), with nine other isolates. Groups **B** and **C** each contained three isolates and the remainder of the isolates had unique patterns. The diversity of fingerprints was high in this method and there were no significant common ERIC markers between these isolates (**Figure 3.6**).

3.3.2.3 *PCR-ribotyping*

PCR-Ribotyping of the isolates gave fingerprints with bands ranging from <0.4-1.0 kb (**Figure 3.7**). This method produced the simplest patterns which were easy to interpret. On the basis of four distinct bands, eight groups were recognised for the 29 isolates and each was assigned a letter (lower case) (**Table 3.5**). Group '**a**' included the type strain (THs) and ten other isolates, group '**b**' included eight isolates. The group '**c**' and '**d**' included three isolates in each. The remaining isolate showed a unique banding pattern for PCR-ribotyping. All isolates showed common markers of 0.4 and 0.7 kb.

For each typing method, many of the isolates produced the same pattern as the type strain (11 isolates for REP- PCR, 10 for ERIC-PCR and 11 for PCR-ribotyping) and all of these whose site of isolation was known were lung isolates (**Table 3.6**). The identical patterns obtained are shown for the type strain and three of the isolates in **Figure 3.8**. However, not all the same group and, indeed, isolates SA04, SA07, SA13, SA48 and SA49 produced different fingerprints for each typing method (**Table 3.6**). Strains SA21, SA22 and SA23 were genital isolates from different animals within the same herd and which had a history of subnormal fertility. They were indistinguishable by all three methods (**Table 3.6**). Although, many of respiratory isolates showed the same fingerprints, genital isolates showed heterogenous patterns. Genital isolates

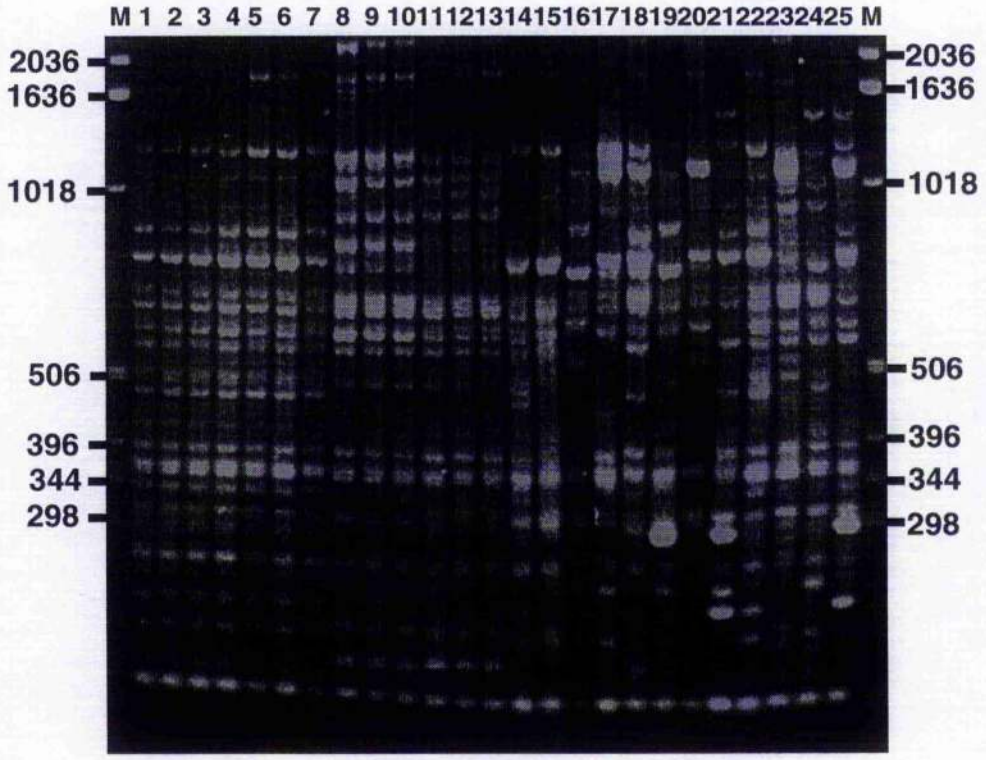

Figure 3.6 ***Fingerprints obtained by ERIC-PCR for H. somnus isolates.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-15, 17, 18 and 20-25: *H. somnus* isolates, THs (type strain), SA05, SA06, SA14, SA15, SA19, SA20, SA01, SA02, SA03, SA21, SA22, SA23, SA07, SA13, SA04, V3, V8, X4, SA17, SA12, X1 and SA11 respectively; lanes 16 and 19: *H. ovis* isolates SA16 and SA08 respectively. Isolates SA48, SA49, SA50, SA51, SA52, SA68 are not included in this figure. The profiles have been arranged so that isolates of a similar type are grouped together. Lanes 1-7 (type A), lanes 8-10 (type B), lanes 11-13 (type C), lane 14 (type D), lane 15 (type E), lane 17 (type G), lane 18 (type H), lane 20 (type J), lane 21 (type K), lane 22 (type F), lane 23 (type M), lane 24 (type N) and lane 25 (type O) (see **Table 3.5**).

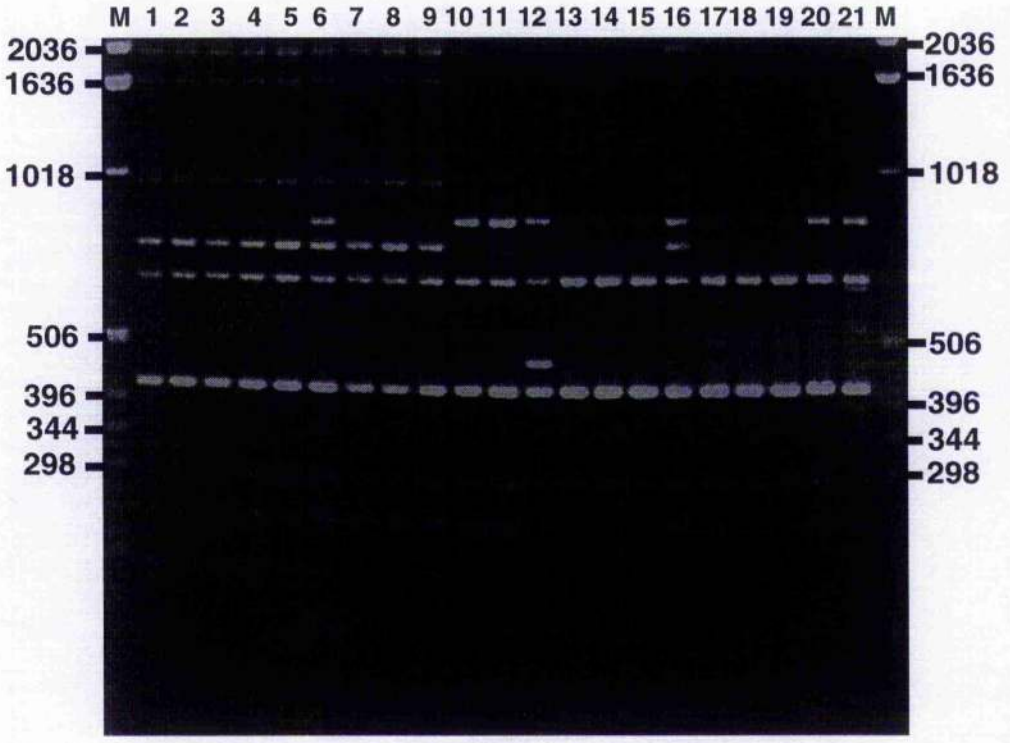

Figure 3.7 ***Fingerprints obtained by PCR-ribotyping for H. somnus isolates.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-20: *H. somnus* isolates, THs (type strain), SA05, SA06, SA14, SA15, SA07, SA17, SA19, SA20, SA12, V3, X1, SA01, SA02, SA03, SA11, SA21, SA22, SA23 and SA13 respectively; lane 21: *H. ovis* isolate SA08. Isolates SA04, SA16, SA48, SA49, SA50, SA51, SA52, SA68, V8 and X4 are not included in this figure. The profiles have been arranged so that, in general isolates of a similar type are grouped together. Lanes 1-5 and 7-9 (type a), lanes 6 and 16 (type d), lanes 10, 11 and 20 (type c), lane 12 (type f), lanes 13-15 and 17-19 (type b) (see **Table 3.5**).

Table 3.6 ***Distribution of H. somnus isolates among PCR types***

Isolate	Source	Site of isolation	Disease status	Geographic origin	REP Type	ERIC Type	Ribo Type
THs*	Bovine	No record	No record	No record	1	A	a
SA05	Bovine	Lung	No record	Dumfries	1	A	a
SA06	Bovine	Lung	Pneumonic	Ayr	1	A	a
SA14	Bovine	Lung	Pneumonic	Aberdeen	1	A	a
SA15	Bovine	Lung	Pneumonic	Ayr	1	A	a
SA19	Bovine	Lung	Pneumonic	Aberdeen	1	A	a
SA20	Bovine	Lung	Pneumonic	Aberdeen	1	A	a
SA50	Bovine	Lung	Pneumonic	Aberdeen	1	A	a
SA51	Bovine	No record	No record	Edinburgh	1	A	a
SA07	Bovine	Lung	Pneumonic	Aberdeen	1	D	d
SA17	Bovine	Lung	Normal	Aberdeen	1	F	a
SA01	Bovine	No record	No record	No record	2	B	b
SA02	Bovine	No record	No record	No record	2	B	b
SA03	Bovine	No record	No record	No record	2	B	b
SA21[a]	Bovine	prepuce	Subfertile	Glasgow	3	C	b
SA22[a]	Bovine	prepuce	Subfertile	Glasgow	3	C	b
SA23[a]	Bovine	prepuce	Subfertile	Glasgow	3	C	b
SA04	Bovine	Lung	Pneumonic	Dumfries	4	G	b
SA52	Bovine	Vagina	Inflamed	Edinburgh	4	I	e
SA68	Bovine	Lung	Pneumonic	Edinburgh	4	P	b
X4	Bovine	Vagina	Culled	No record	5	K	d
SA49	Bovine	Lung	Pneumonic	Aberdeen	5	L	h
SA48	Bovine	Lung	Pneumonic	Aberdeen	6	A	a
SA12	Bovine	semen	Normal	St. Boswells	6	M	c
SA11	Bovine	semen	Normal	St. Boswells	7	O	d
SA13	Bovine	Lung	Pneumonic	Aberdeen	8	E	c
X1	Bovine	Cervix	Culled	No record	9	N	f
V3	Bovine	Vestibular opening	Culled	No record	10	H	c
V8	Bovine	Cervix	Culled	No record	11	J	g

* THs: ATCC type strain.

[a] Isolated from different animals of the same herd that showed subnormal fertility.

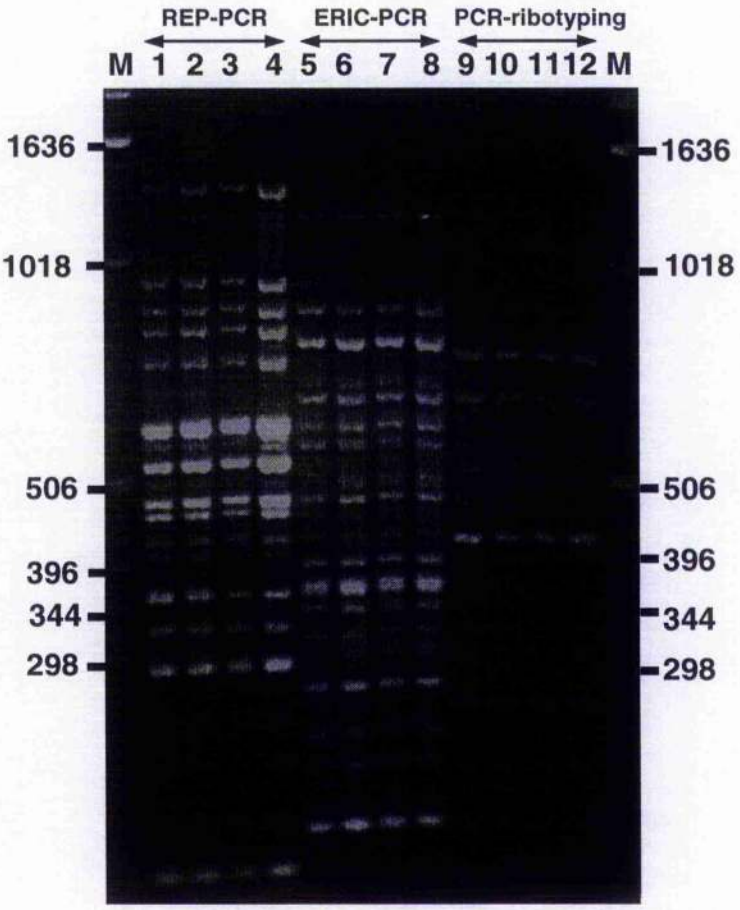

Figure 3.8 ***Similarity of certain H. somnus strains by each typing method.*** The type strain and three lung isolates (SA05, SA06 and SA14) were analysed by each PCR method. Lanes M: 1 Kb DNA Ladder. Lanes 1-4, REP-PCR; lanes 5-8, ERIC-PCR; and lanes 9-12, PCR-ribotyping. Lanes 1, 5 and 9, Type strain; lanes 2, 6 and 10, SA05; lanes 3, 7 and 11, SA06; and lanes 4, 8 and 12, SA14 respectively.

were also examined from slaughterhouse samples taken from individual animals which were not necessarily from the same herd. Isolates from different sites (e.g. cervix and vestibular opening) in any one animal gave the same REP-PCR profile, but the profiles differed between isolates from different animals (**Figure 3.9**).

3.3.3 Fingerprinting of *H. ovis*

3.3.3.1 *REP-PCR*

This method produced 11 distinguishable patterns for the 19 *H. ovis* isolates and, therefore the highest degree of discrimination between isolates (**Table 3.7**). The banding patterns were more complex than those seen with the ERIC-PCR and PCR-ribotyping methods with bands ranging in size from 0.25-2.5 kb and of various intensities (**Figure 3.10**). The 19 isolates were grouped into 11 distinct patterns of fingerprints, on the basis of 10 distinct bands and each pattern was assigned a number (**Table 3.7**). Group **1** included four isolates, group **2** and **3** had three isolates each and group **4** had two isolates with the remaining seven isolates, including SA24 (Low and Graham, 1985) used as the reference isolate in the study, showing unique banding patterns. There were REP markers of 0.35, 0.4, 0.52, 0.75, 0.95 and 1.0 kb common to all *H. ovis* isolates.

3.3.3.2 *ERIC-PCR*

With this method, profiles of *H. ovis* revealed amplified bands ranging from 0.075-0.95 kb with various intensities (**Figure 3.11**) and seven of these were used for typing. The fingerprints obtained by this method for *H. somnus* were much more complex than those for *H. ovis*. The distribution of isolates were: Group **A**, four isolates, group **B**, seven isolates, group **C**, three isolates, group **D**, two isolates and groups **E**, **F** (isolate SA24) and **G** contained one isolate each (**Table 3.7**). ERIC-PCR fingerprints showed common markers with bands at 0.15 and 0.75 kb being the most intense (**Figure 3.11**).

3.3.3.3 *PCR-ribotyping*

PCR-ribotyping of the 19 *H. ovis* isolates gave fingerprints with bands ranging from 0.215-1.018 kb (**Figure 3.12**). As for *H. somnus* this method produced the simplest patterns of the three and were easy to interpret. Six distinct bands were used to determine the fingerprint. Five groups were recognised for the 19 isolates of *H. ovis* (**Table 3.7**).

Figure 3.9 ***Comparison of different isolates from slaugterhouse materials by REP-PCR.*** Lanes M: 1 Kb DNA Ladder. Lane 1: *H. somnus* THs (type strain). Lanes 2 and 3: isolates (X1) from cervix and vestibular opening of the same animal. Lanes 4 and 5: isolates (X4) from cervix and vestibular opening of another animal. Lanes 6-16 represent different colonies taken from the same culture plate from which isolate X4 (lane 4) was isolated. Lane 17: *A. seminis* (X16) isolate. Lanes 18, 19, 20 and 21 (V3) represent isolates from different sites of another animal: lanes 18 and 19 were different sized colonies from the cervix, lane 20 was an isolate from the vestibular opening and lane 21 was an isolate from the uterus. Lanes 22 and 23 (V8) are isolates from another animal but isolated from the cervix and the vagina respectively.

Table 3.7 *Types of H. ovis isolates by three different typing methods*

REP types (11 types)	Isolates	No. of isolates
1	SA29, SA45, SA46, SA73	4
2	SA16, SA27, SA44	3
3	SA56, SA57, SA58	3
4	SA26, SA55	2
5	SA28	1
6	SA54	1
7	SA69	1
8	SA72	1
9	SA53	1
10	SA24	1
11	SA08	1

ERIC types (7 types)	Isolates	No. of isolates
A	SA29, SA45, SA46, SA73	4
B	SA16, SA26, SA27, SA44, SA55, SA28, SA54	7
C	SA56, SA57, SA58	3
D	SA69, SA72	2
E	SA53	1
F	SA24	1
G	SA08	1

Ribotypes (5 types)	Isolates	No. of isolates
a	SA08, SA16, SA29, SA45, SA46, SA73	6
b	SA26, SA27, SA28, SA44, SA54, SA55	6
c	SA56, SA57, SA58	3
d	SA53, SA69, SA72	3
e	SA24	1

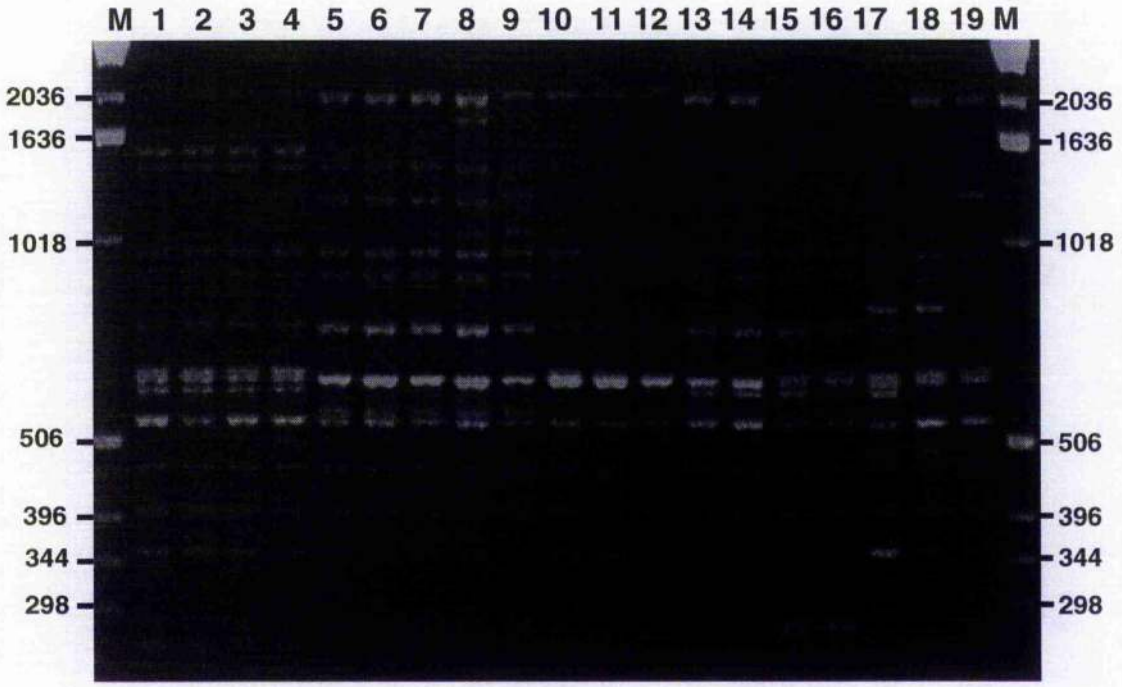

Figure 3.10 ***Fingerprints obtained by REP-PCR for H. ovis isolates.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-19: *H. ovis* isolates, SA29, SA45, SA46, SA73, SA27, SA44, SA16, SA28, SA54, SA56, SA57, SA58, SA26, SA55, SA69, SA72, SA53, SA24 and SA08 respectively. The profiles have been arranged so that isolates of a similar type are grouped together. Lanes 1-4 (type 1), lanes 5-7 (type 2), lane 8 (type 5), lane 9 (type 6), lanes 10-12 (type 3), lanes 13-14 (type 4), lane 15 (type 7), lane 16 (type 8), lane 17 (type 9), lane 18 (type 10) and lane 19 (type 11) (see **Table 3.7**).

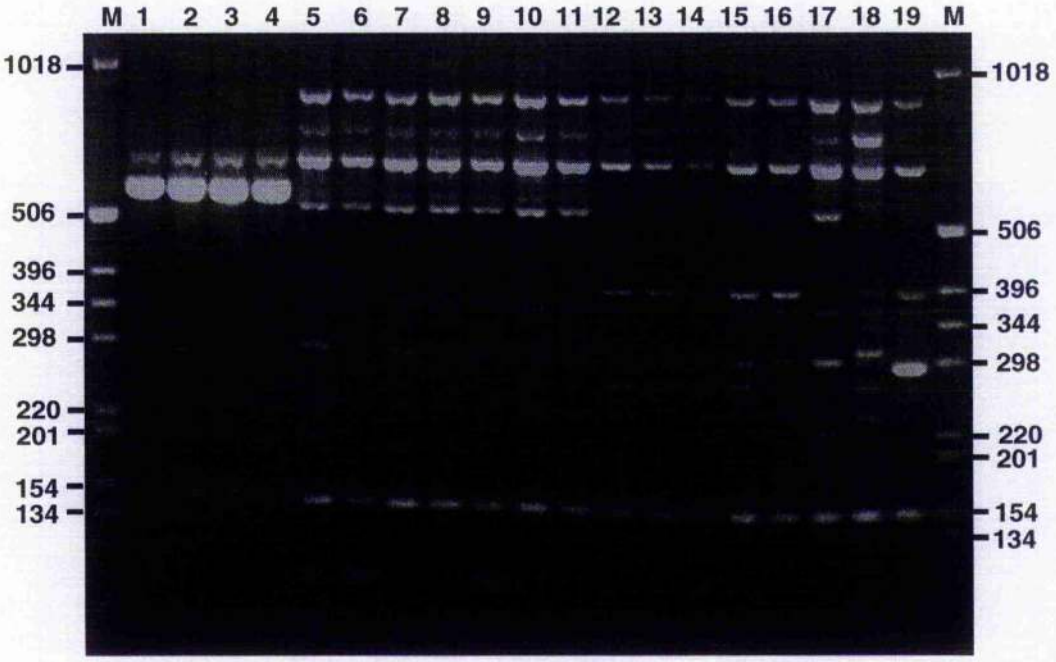

Figure 3.11 ***Fingerprints obtained by ERIC PCR for H. ovis isolates.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-19: *H. ovis* isolates, SA29, SA45, SA46, SA73, SA27, SA44, SA26, SA55, SA16, SA28, SA54, SA56, SA57, SA58, SA69, SA72, SA53, SA24 and SA08 respectively. The profiles have been arranged so that isolates of a similar type are grouped together. Lanes 1-4 (type A), lanes 5-11 (type B), lanes 12-14 (type C), lanes 15-16 (type D), lane 17 (type E), lane 18 (type F) and lane 19 (type G) (see **Table 3.7**).

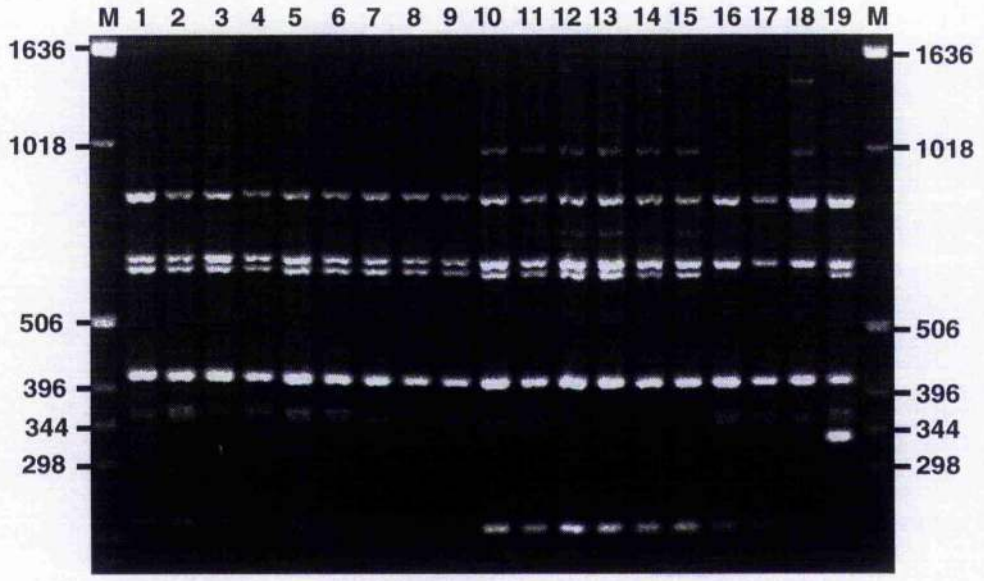

Figure 3.12 ***Fingerprints obtained by PCR ribotyping for H. ovis isolates.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-19: *H. ovis* isolates, SA08, SA26, SA16, SA73, SA45, SA46, SA56, SA57, SA58, SA55, SA26, SA27, SA28, SA44, SA54, SA69, SA72, SA53 and SA24 respectively. The profiles have been arranged so that isolates of a similar type are grouped together. Lanes 1-6 (type **a**), lanes 7-9 (type **b**), lanes 10-15 (type **c**), lanes 16-18 (type **d**) and lane 19 (type **e**) (see **Table 3.7**).

Groups 'a' and 'b' each included six isolates. Groups 'c' and 'd' each included three isolates and the remaining isolate (SA24) showed a unique banding pattern. The PCR-ribotyping patterns for *H. ovis* showed three common markers of 0.4 , 0.7 and 0.9 kb.

The *H. ovis* isolates tested showed clear differentiation by all three PCR methods. The reference isolate SA24 (Low and Graham, 1985) showed unique patterns for all three methods (**Table 3.7**). In general, there was a close correlation between the typing methods. For example, isolates SA29, SA45, SA46 and SA73 comprised type 1 of REP-PCR, type A of ERIC-PCR and type 'a' of PCR-ribotyping (**Table 3.8**). The three isolates (SA56, SA57 and SA58) from a flock which had subnormal fertility, produced the same fingerprints for all typing methods.

3.3.4 Comparison of *H. somnus* and *H. ovis* by PCR methods

Comparison of **Figures 3.7** and **3.12** shows that PCR-ribotyping patterns of *H. somnus* isolates were clearly differentiated from those of *H. ovis* because the latter had an extra unique band close to the PCR-ribotyping marker of 0.7 kb except for three *H. ovis* isolates SA53, SA69 and SA72 (**Figure 3.12**, lanes 16, 17 and 18). These latter strains showed profiles similar to the PCR-ribotype profile 'c' obtained with *H. somnus* bovine strains SA12, SA13 and V3 (**Figure 3.13**). Two of these ovine isolates showed similar banding patterns by ERIC-PCR (SA69 and SA72) (**Figure 3.11**, lanes 15 and 16) but they showed unique patterns by REP-PCR. REP- and ERIC-PCR fingerprints did not show clear differences between isolates of *H. somnus* and *H. ovis* because the REP and ERIC types of these two species showed considerable variation, as shown in **Figure 3.14** and **Figure 3.15** respectively. **Figure 3.14** shows a side-by-side comparison of representatives of each REP type of *H. somnus* and *H. ovis* and **Figure 3.15** shows representatives of each ERIC-type of these two species.

3.3.5 Fingerprinting of *A. seminis*

3.3.5.1 *REP-PCR*

With the REP-PCR method, profiles of *A. seminis* revealed amplified bands ranging from <0.25-2.5 kb with various intensities (**Figure 3.16**), of which 18 were used to assign fingerprints. This method produced more complex banding patterns than those seen

Table 3.8 ***Distribution of H. ovis isolates among PCR types***

Isolate	Source	Site of isolation	Disease status	Geographic origin	REP type	ERIC type	Ribo type
SA29	Ovine	Semen	Fertile	No records	1	A	a
SA45	Ovine	Semen	No record	South Scotland	1	A	a
SA46	Ovine	Semen	Fertile	Central Scotland	1	A	a
SA73	Ovine	Semen	No records	North England	1	A	a
SA16	Ovine	Semen	No records	No records	2	B	a
SA27	Ovine	Semen	Subfertile	North England	2	B	b
SA44	Ovine	Semen	Fertile	No records	2	B	b
SA56[b]	Ovine	Vagina	Subfertile	Central Scotland	3	C	c
SA57[b]	Ovine	Vagina	Subfertile	Central Scotland	3	C	c
SA58[b]	Ovine	Vagina	Subfertile	Central Scotland	3	C	c
SA26	Ovine	Semen	Fertile	Central Scotland	4	B	b
SA55	Ovine	Semen	Infertile	North Scotland	4	B	b
SA28	Ovine	Semen	Subfertile	North England	5	B	b
SA54	Ovine	Semen	Subfertile	Central Scotland	6	B	b
SA69	Ovine	Semen	Fertile	South Scotland	7	D	d
SA72	Ovine	Prepuce	Fertile	Central Scotland	8	D	d
SA53	Ovine	Semen	Infertile	Central Scotland	9	E	d
SA24[*]	Ovine	Semen	Infertile	South Scotland	10	F	e
SA08	Ovine	Semen	No records	No records	11	G	a

[*] SA24: reference isolate (Low and Graham, 1985).

[b] Isolated from different animals of the same flock that showed subnormal fertility.

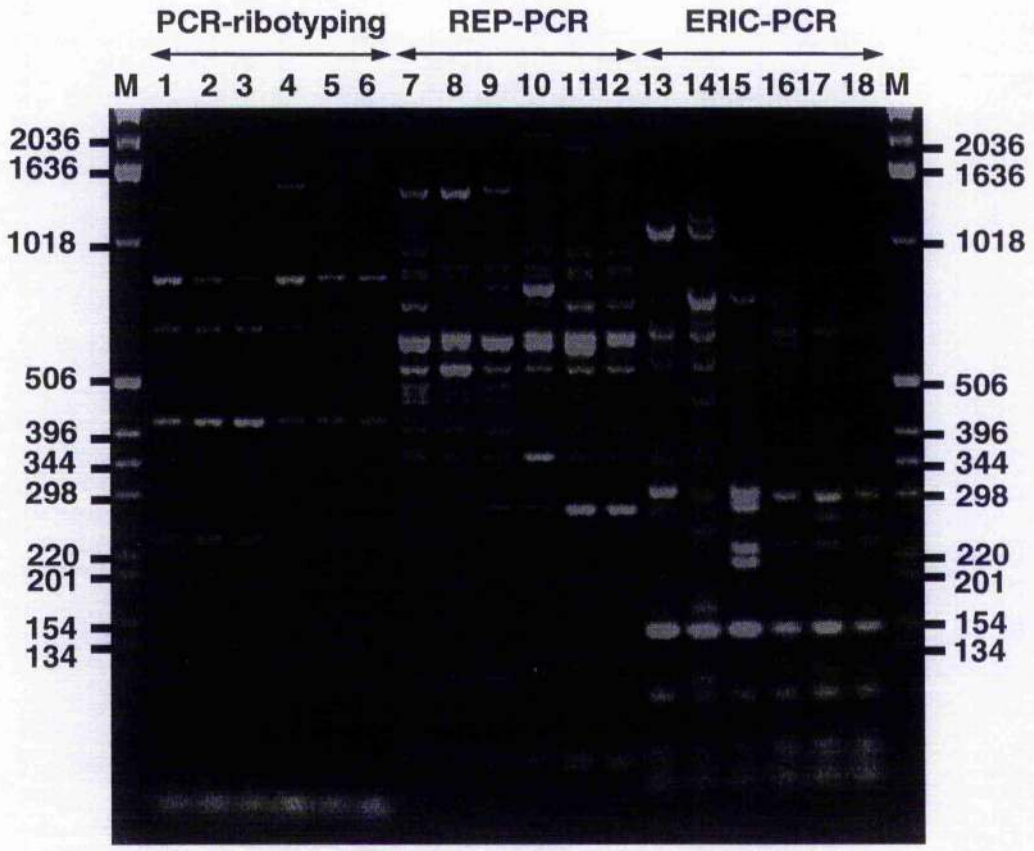

Figure 3.13 ***Comparison of the profiles of three ovine isolates (H. ovis, lanes 4-6, 10-12, 16-18) with those of three bovine isolates (H. somnus, lanes 1-3, 7-9, 13-15) by the three PCR-fingerprinting methods.*** Lanes. M: 1 Kb DNA Ladder. Lanes 1-6: PCR-ribotyping, lanes 7-12: REP-PCR and lanes 13-18: ERIC-PCR. *H. somnus*: strain SA12 (lanes 1, 7, 13); V3 (lanes 2, 8, 14); X4 (lanes 3, 9, 15). *H. ovis*: strains SA53 (lanes 4, 10, 16): SA69 (lanes 5, 11, 17); SA72 (lanes 6, 12, 18).

Figure 3.14 ***Comparison of different REP types of H. somnus and H. ovis.*** Lanes M: 1 Kb marker. *H. somnus* lanes 1-11: THs (type 1), SA01 (type 2), SA21 (type 3), SA11 (type 7), SA12 (type 6), SA13 (type 8), X1 (type 9), V3 (type 10), SA04 (type 4), V8 (type 11) and X4 (type 5) respectively. *H. ovis* lanes 1-9: representative isolates of with different profiles for REP-PCR: SA69 (type 7), SA72 (type 8), SA53 (type 9), SA29 (type 1), SA16 (type 2), SA56 (type 3), SA24 (type 10) and SA08 (type 11). The central marker lane (M) has been inserted electronically.

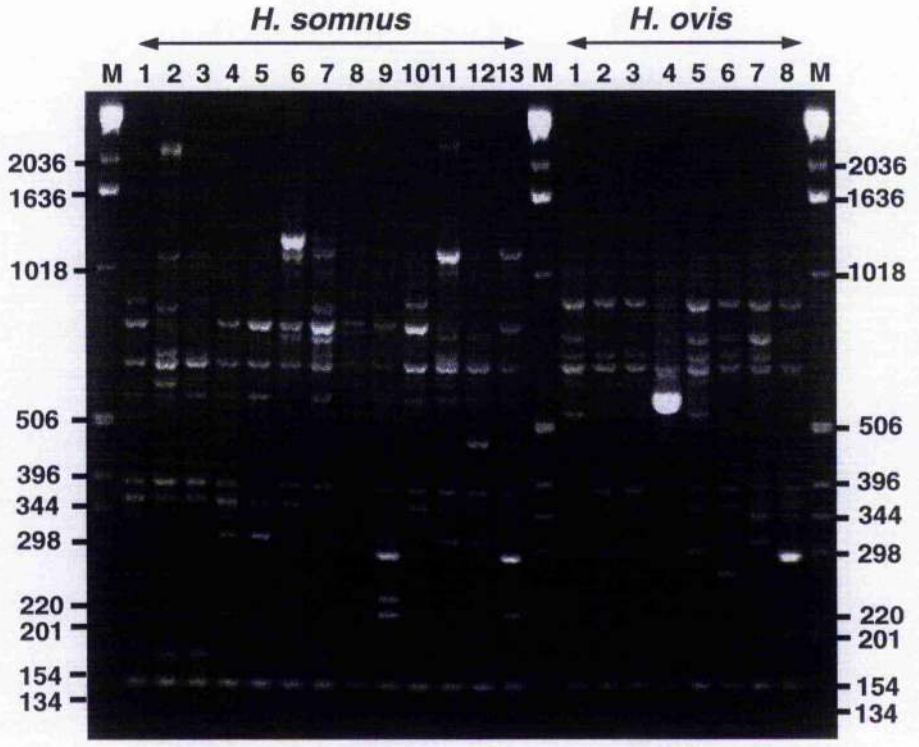

Figure 3.15 ***Comparison of different ERIC types of H. somnus and H. ovis.*** Lanes M: 1 Kb marker. *H. somnus* lanes 1-13: THs (type A), SA01 (type B), SA21 (type C), SA07 (type D), SA13 (type E), SA04 (type G), V3 (type H), V8 (type J), X4 (type K), SA17 (type F), SA12 (type M), X1 (type N) and SA11 (type O) respectively. The ERIC fingerprints of SA49 and SA68 are not shown. *H. ovis* lanes 1-8: SA53 (type E), SA69 (type D), SA72 (type D), SA29 (type A), SA16 (type B), SA56 (type C), SA24 (type F) and SA08 (type G). The central marker lane (M) has been inserted electronically.

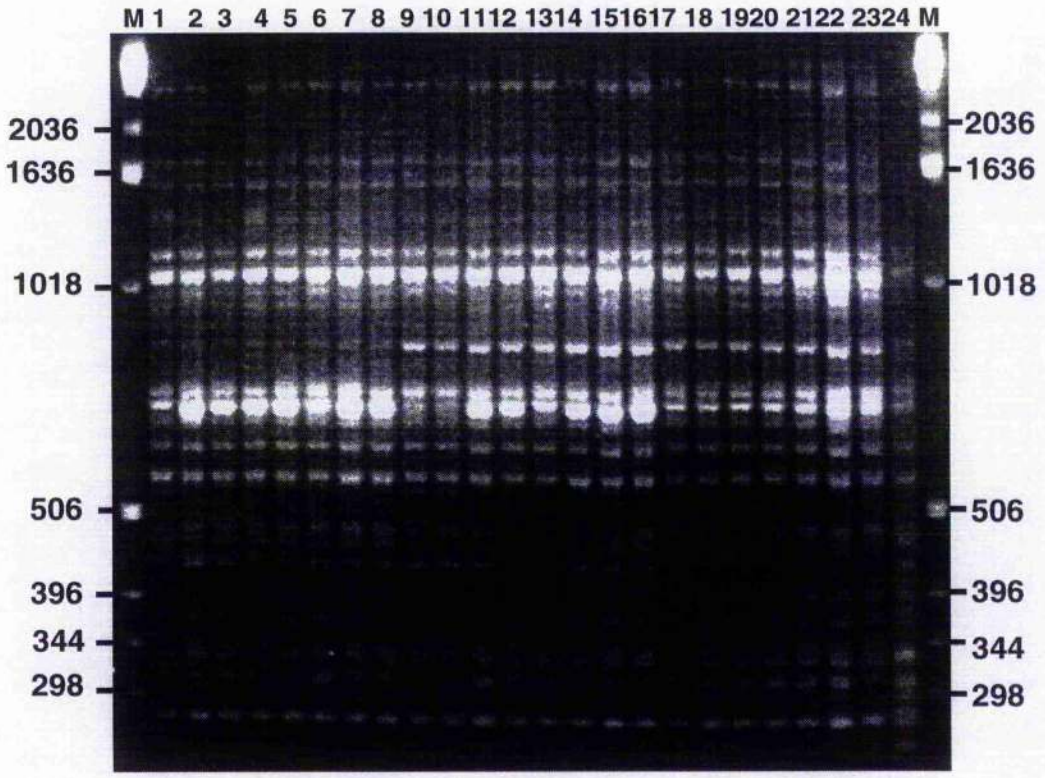

Figure 3.16 ***Fingerprints obtained by REP-PCR for A. seminis isolates.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-24: *A. seminis* isolates, type strain (TAs), SA25, SA31, SA32, SA36, SA39, SA67, SA71, SA65, SA66, SA33, SA37, SA43, SA60, SA63, SA70, SA30, SA34, SA35, SA38, SA61, SA62, SA64 and X16 respectively. The profiles have been arranged so that isolates of a similar type are grouped together. Lanes 1-8 (type 1), lanes 9-10 (type 4), lanes 11-16 (type 3), lanes 17-23 (type 2), lane 24 (type 5) (see **Table 3.9**).

with the PCR ribotyping method and were more difficult to interpret, especially when less intense bands were present. However, the 24 isolates were grouped into 5 distinct patterns of fingerprints, each of which was assigned a number (**Table 3.9**). Group **1** included the type strain and seven other isolates with similar patterns and this was the largest group. Group **2** had seven isolates, group **3** had six isolates and group **4** had two isolates. The bovine strain, X16, had a unique pattern. There were major common REP markers of 0.275, 0.55, 0.6, 0.8, 1.050 and 1.1 kb common to all *A. seminis* isolates. By REP-PCR, the bovine isolate, X16 was clearly distinct from all other strains.

3.3.5.2 *ERIC-PCR*

This method produced 9 distinguishable but complex patterns for the 24 isolates and, therefore the highest degree of discrimination between isolates (**Table 3.9**). The fragment sizes ranged from <0.1-1.65 kb with various band intensities (**Figure 3.17**). Eighteen bands were used to assign fingerprints. The distribution of isolates were: type **A**, nine isolates, type **B**, five isolates including the type strain, TAs, type **C**, four isolates and the rest of the types each contained one isolate. ERIC-PCR fingerprints also showed common markers with bands at 0.33, 0.515 and 0.6 kb being the most intense (**Figure 3.17**). The bovine isolate, X16, was clearly distinguishable from all other strains by ERIC-PCR.

3.3.5.3 *PCR-ribotyping*

PCR-ribotyping of the 24 isolates gave very similar fingerprints for all isolates except isolate SA33. The profiles were characterised by two high intensity bands of 0.55 and 0.7 kb (**Figure 3.18**) and very low intensity bands of 0.85, 1.0 and 1.6 kb. Isolate SA33 showed a comparatively intense additional band of 0.95 kb. For this reason, this isolate was considered to be a separate type by PCR-ribotyping (**Table 3.9**). The single isolate of bovine origin showed the same band pattern as that of the ovine isolates (**Figure 3.18,** lane 22).

In general, *A. seminis* isolates produced fewer types by all three PCR method (5 types for REP-PCR, 9 type for ERIC-PCR and only 2 types for PCR-ribotyping (**Table 3.9**) when compared with the other two species. The *A. seminis* type strain showed a unique combination of the types but the PCR profiles were similar to those of some other

Table 3.9 *Types of A. seminis isolates by three different typing methods*

REP type (5 types)	Isolates	No. of isolates
1	TAs, SA25, SA31, SA32, SA36, SA39, SA67, SA71	8
2	SA30, SA34, SA35, SA38, SA61, SA62, SA64	7
3	SA33, SA37, SA43, SA60, SA63, SA70	6
4	SA65, SA66	2
5	X16	1

ERIC type (9 types)	Isolates	No. of isolates
A	SA30, SA33, SA34, SA35, SA38, SA43, SA61, SA62, SA64	9
B	TAs, SA37, SA60, SA65, SA66	5
C	SA31, SA36, SA67, SA71	4
D	SA70	1
E	SA39	1
F	SA25	1
G	SA32	1
H	SA63	1
I	X16	1

Ribotype (2 types)	Isolates	No. of isolates
a	TAs, SA25, SA30, SA31, SA32, SA34, SA35, SA36, SA37, SA38, SA39, SA43, SA60, SA61, SA62, SA63, SA64, SA65, SA66, SA67, SA70, SA71, X16	23
b	SA33	1

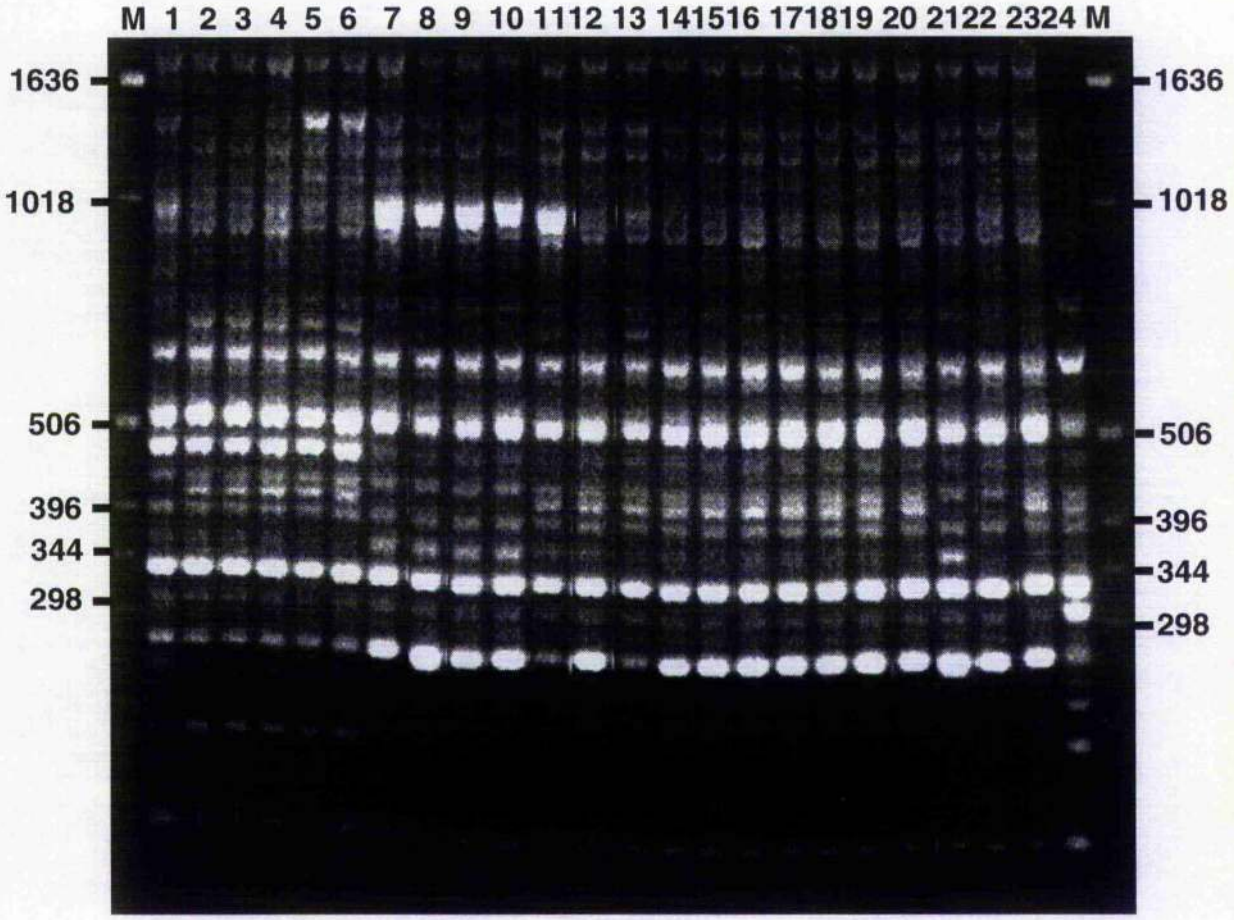

Figure 3.17 ***Fingerprints obtained by ERIC-PCR for A. seminis isolates.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-24: *A. seminis* isolates, SA39, type strain (TAs), SA37, SA60, SA65, SA66, SA31, SA36, SA67, SA71, SA70, SA30, SA33, SA34, SA35, SA38, SA43, SA61, SA62, SA64, SA25, SA32, SA63 and X16 respectively. The profiles have been arranged electronically so that isolates of a similar type are grouped together. Lane 1 (type E), lanes 2-6 (type B), lanes 7-10 (type C), lane 11 (type D), lanes 12-20 (type A), lane 21 (type F), lane 22 (type G), lane 23 (type H) and lane 24 (type I) (see **Table 3.9**).

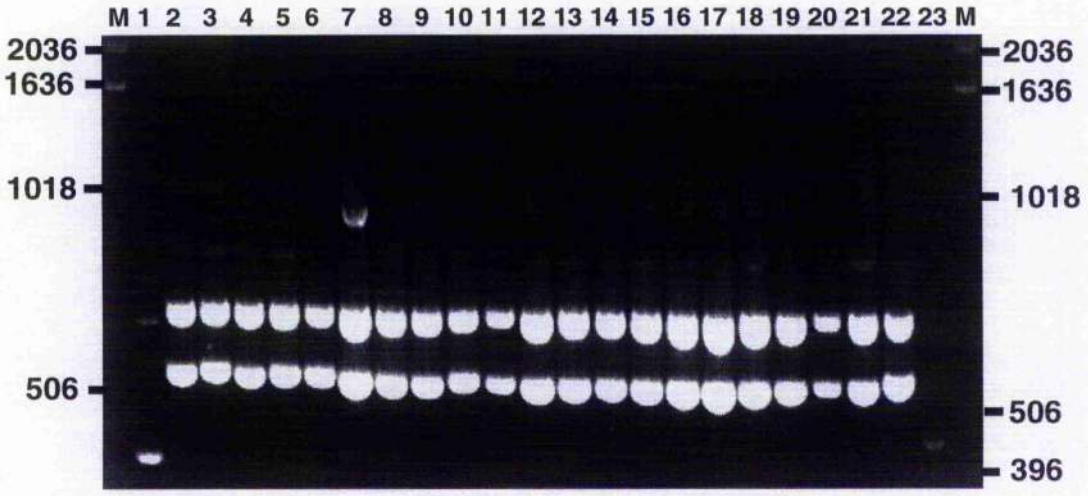

Figure 3.18 ***Fingerprints obtained by PCR-ribotyping A. seminis isolates.*** Lanes M: 1 Kb DNA Ladder (Gibco BRL, UK). Lane 1: *H. somnus*. Lanes 2-22: *A. seminis* strains TAs (type strain), SA25, SA30, SA31, SA32, SA33, SA35, SA37, SA38, SA39, SA43, SA60, SA61, SA62, SA63, SA64, SA66, SA67, SA70, SA71 and X16 respectively. *A. seminis* isolates SA34, SA36, SA65 are not included but had the same pattern as the other *A. seminis* isolates except SA33 (**Table 3.9**). Lane 23: *A. actinomycetemcomitans.*

strains (**Table 3.10**). The *A. seminis* isolates obtained from sequential samples from the same animal at different times produced identical fingerprints (SA30 and SA64; SA34 and SA38; SA65 and SA66, **Table 3.10**). The PCR types did not show clear correlation with breed of ram or disease status but most of the South Scotland isolates showed the same combination of types. The bovine strain X16 showed unique patterns for REP- and ERIC-PCR but the PCR-ribotyping pattern was the same as that of most of the *A. seminis* isolates (**Table 3.10**).

3.3.6 Differentiation of *A. seminis* from *H. somnus* and *H. ovis* by PCR methods

The fingerprints generated by the three PCR methods showed that *A. seminis* was clearly different from *H. somnus* and *H. ovis*. Different isolates of *H. ovis* and *A. seminis* showed clear differences in fingerprints by all three methods as shown with representative strains of each species in **Figure 3.19**. With its simplicity of pattern, the PCR-ribotyping method readily differentiated all three species as shown for representative strains in **Figure 3.20**. *H. somnus* was differentiated from *H. ovis* at around 0.7 kb where it had a single amplimer band compared with the double band of the *H. ovis* isolates. The *A. seminis* isolates were differentiated by two bold bands at 0.55 kb and 0.7 kb (**Figure 3.20**).

3.3.7 Differentiation of from *H. somnus*, *H. ovis* and *A. seminis* from other bacteria

With other, related bacteria, *A. seminis, A. pleuropneumoniae, P. haemolytica, P. trehalosi, P. multocida* and other unidentified isolates from the bovine reproductive tract, REP-PCR, ERIC-PCR and PCR-ribotyping produced completely different patterns for each species. This is illustrated for REP-PCR in **Figure 3.21** and PCR-ribotyping in **Figure 3.22**.

3.3.8 Reproducibility of PCR fingerprints

The reproducibility of banding patterns was generally excellent when the same batch of reagents was used although some minor, day-to-day variation in intensity was observed with less intense amplimers. In general, there was no effect on the PCR fingerprints with the change of template preparations i.e. when different batches of boiled cell extracts were

Table 3.10 ***Distribution of A. seminis isolates among PCR types***

Isolate	Source	Breed of ram	Disease status	Geographic origin	REP type	ERIC type	Ribo type
TAs*	Ovine	No record	Epididymitis	Australia	1	B	a
SA31	Ovine	Suffolk	Subfertile	North Scotland	1	C	a
SA36	Ovine	Scottish Blackface	Normal	South Scotland	1	C	a
SA67	Ovine	Border Leicester	Normal	North Scotland	1	C	a
SA71	Ovine	No record	Normal	No record	1	C	a
SA39	Ovine	Suffolk	Normal	South Scotland	1	E	a
SA25	Ovine	No record	No record	No record	1	F	a
SA32	Ovine	Suffolk	Epididymitis	England	1	G	a
SA30[c]	Ovine	Suffolk	Normal	South Scotland	2	A	a
SA34[d]	Ovine	Suffolk	Epididymitis	South Scotland	2	A	a
SA35	Ovine	Texel	Normal	South Scotland	2	A	a
SA38[d]	Ovine	Suffolk	Epididymitis	South Scotland	2	A	a
SA61	Ovine	Texel	Epididymitis	South Scotland	2	A	a
SA62	Ovine	Poll Dorset	Normal	South Scotland	2	A	a
SA64[c]	Ovine	Suffolk	Normal	South Scotland	2	A	a
SA43	Ovine	Texel	Epididymitis	South Scotland	3	A	a
SA33	Ovine	Poll Dorset	Epididymitis	England	3	A	b
SA37	Ovine	Suffolk	Epididymitis	South Scotland	3	B	a
SA60	Ovine	Suffolk	Epididymitis	South Scotland	3	B	a
SA70	Ovine	Border Leicester	Epididymitis	North Scotland	3	D	a
SA63	Ovine	Berrichon de Cher	Normal	England	3	H	a
SA65[e]	Ovine	Suffolk	Normal	North Scotland	4	B	a
SA66[e]	Ovine	Suffolk	Normal	North Scotland	4	B	a
X16	Bovine	Not applicable	Culled	No record	5	I	a

* TAs: NCTC type strain.

c d e Isolated from samples taken at different times from the same animal.

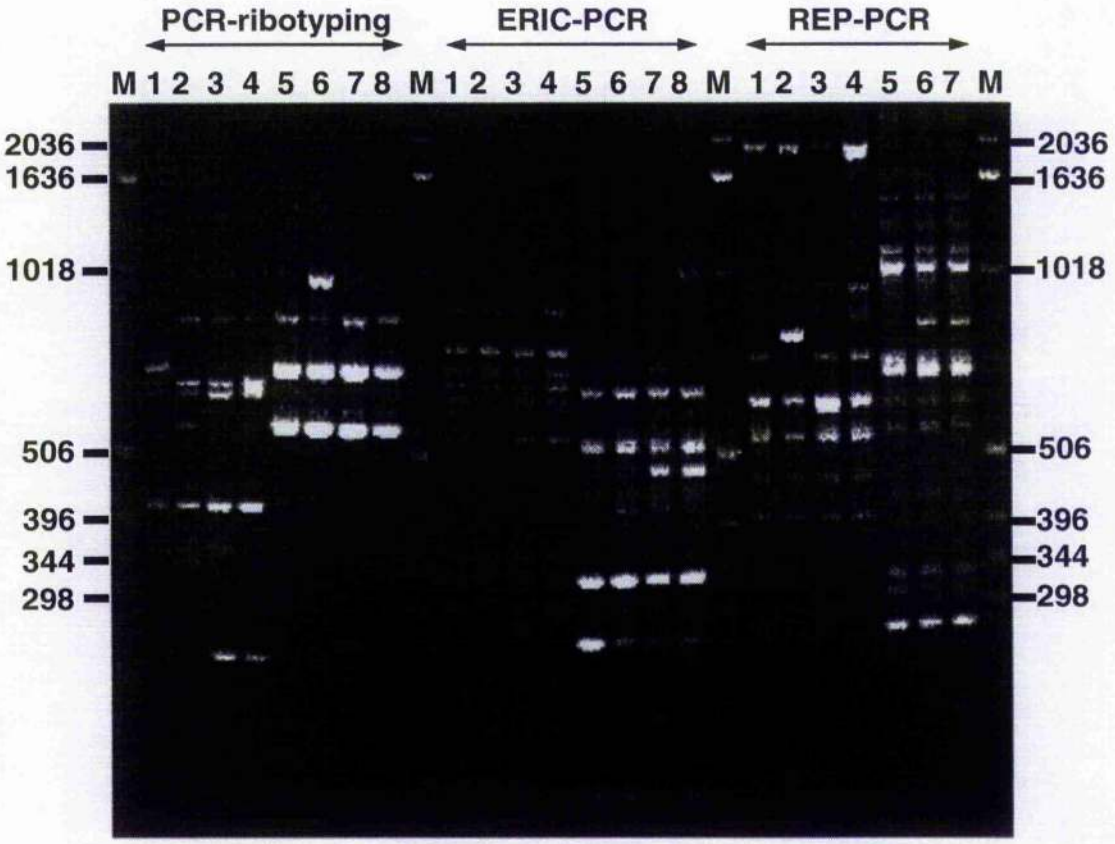

Figure 3.19 ***Differentiation of H. ovis from A. seminis by PCR methods.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-4: different isolates of *H. ovis* SA16, SA24, SA26 and SA44. Lanes 5-8: *A. seminis* strains: SA32, SA33, SA37 and SA39 respectively (Lane 8 of REP-PCR was not included).

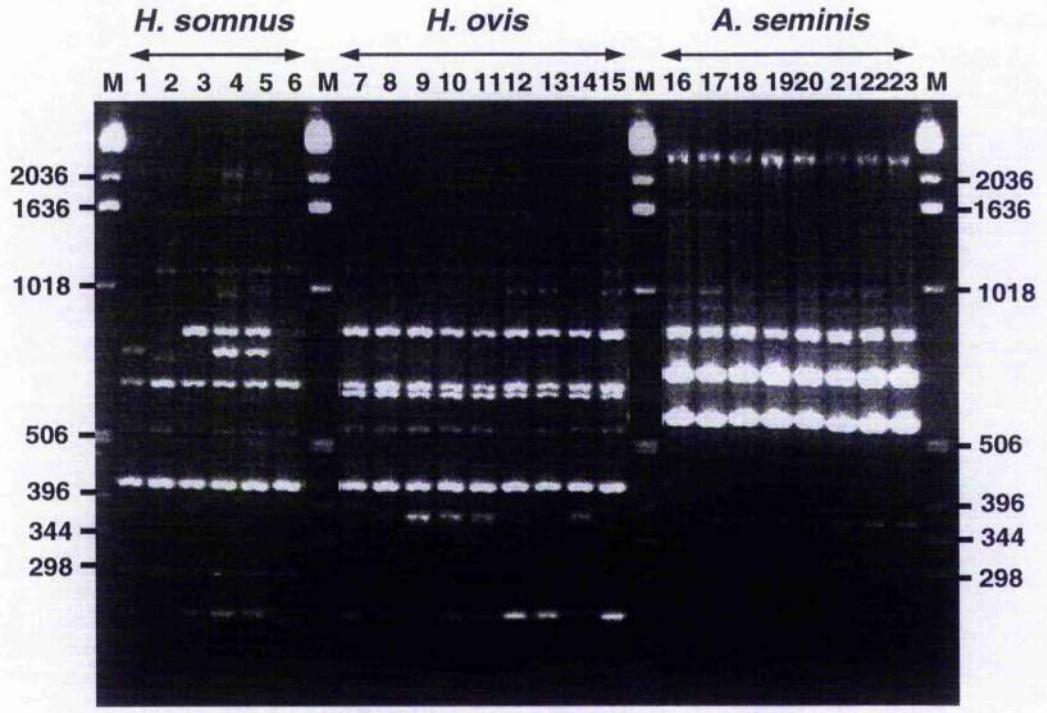

Figure 3.20 ***Comparison of different isolates of H. somnus, H. ovis and A. seminis by PCR-ribotyping.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-6: *H. somnus* isolates THs, SA01, SA12, SA07, X4 and SA04. Lanes 7-15: *H. ovis* isolates SA08, SA16, SA24, SA46, SA45, SA26, SA27, SA29 and SA44. Lanes 16-23: *A. seminis* isolates SA25, TAs, SA31, SA32, SA36, SA37, SA38 and SA39 respectively. The central marker lanes (M) have been inserted electronically.

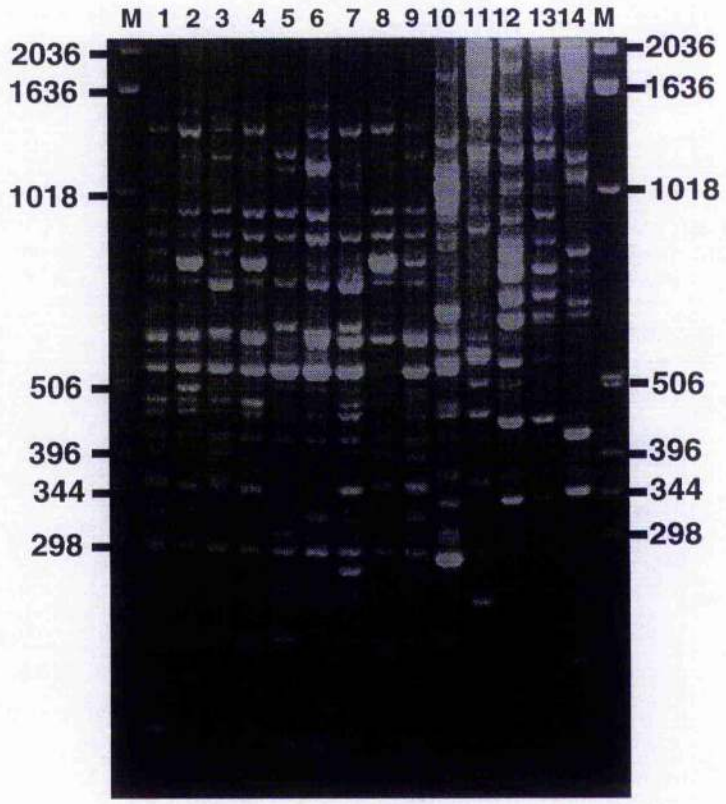

Figure 3.21 ***Comparison of REP-PCR fingerprints of H. somnus with those of some other members of the family Pasteurellaceae.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-9: representatives of the different REP types of *H. somnus* and *H. ovis*; THs, SA01, SA12, SA21, SA08, SA16, SA04, V8 and X4 respectively. Lanes 10-14: *A. seminis*, *A. pleuropneumoniae*, *P. haemolytica*, *P. trehalosi* and *P. multocida* respectively.

Figure 3.22 ***Comparison of PCR-ribotyping fingerprints of H. somnus with those of some other members of the family Pasteurellaceae and those of unidentified isolates.*** Lanes M: 1 Kb DNA Ladder. Lanes 1-4 and 6-7: different REP types of *H. somnus* THs, SA01, SA12, SA07, X1 and SA04 respectively. Lane 5: *H. ovis* SA08. Lane 8-12: *A. seminis* SA31, *A. pleuropneumoniae, P. haemolytica, P. trehalosi* and *P. multocida* respectively. Lanes 13-23: unidentified isolates from bovine and ovine reproductive tracts.

used or when boiled extracts were compared with chromosomal DNA preparations or with different primer batches and primer source and with different batches of *Taq* DNA polymerase. For example, a comparison of profiles obtained for SA08 by PCR-ribotyping on three occasions (lane 21 of **Figure 3.7**, lane 1 of **Figure 3.12** and lane 7 of **Figure 3.20**) are arranged in the composite illustration, **Figure 3.23a**. However, in some instances PCR-ribotyping of *A. seminis* showed non-specific band amplifications like lane 16-23 of **Figure 3.20** and lane 8 of **Figure 3.22** which showed additional bands to those of **Figure 3.18**. This variation has been illustrated in **Figure 3.23b**. These patterns were obtained over an 18 month period using different batches of DNA template preparations, primers, *Taq* DNA polymerase enzyme etc. Because of complexity of banding patterns obtained by ERIC- and REP-PCR and some day-to-day variation in intensity of the bands especially in the minor bands, a direct comparison of pattern differences was difficult between different gels.

The REP-PCR primers REP-IRDT and REP-2DT (**Table 2.4**) however, showed a variation in fingerprints with different primer batches. This was studied in detail as shown in **Table 2.7** and the results with *H. somnus* strains are shown in **Figure 3.24**. The major effect was observed with REP-IRDT primer e.g. lanes 2, 3 and 8, 9 showed similar patterns with primer REP-IRDT from the same batch and primer REP-2DT from different batches. On the other hand, lanes 4, 5, 6, 7 and 10, 11, 12, 13 showed similar patterns (which were different from those obtained with the previous primer combination) with primer REP-2DT from the same batch but with primer REP-IRDT from different batches.

3.3.9 Discriminatory power of PCR methods

In this study, REP-PCR fingerprinting of 29 isolates of *H. somnus* produced 11 distinct profiles, ERIC-PCR produced 16 types and PCR-ribotyping produced eight types. For the 19 isolates of *H. ovis*, 11 types were distinguished by REP-PCR, seven types by ERIC-PCR and five types by PCR-ribotyping. For the 24 isolates of *A. seminis*, five types were identified by REP-PCR, nine types by ERIC-PCR but PCR-ribotyping produced a similar pattern for all isolates except one. The presentation of the number of types in a typing method is not necessarily an expression of the efficiency of the typing method as the distribution of strains between types will depend on the typing methods used. On the other hand, the assessment of the efficency of typing methods should be based on several factors like typability, reproducibility and discrimination. The assessment of typability and reproducibility of a typing method is simple and often they are expressed as a percentage. However, the assessment of discriminatory power of a typing method is complex as it

Figure 3.23a

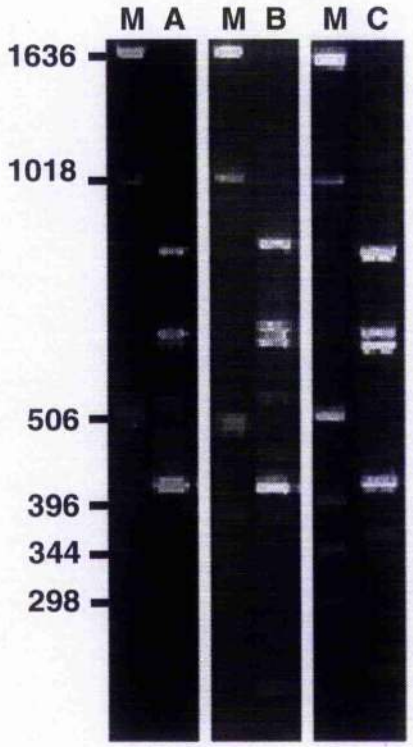

Figure 3.23b

Figure 3.23 ***Composite figures showing the reproducibility of fingerprints.*** (a) shows lane 21 of Figure 3.7 (A), lane 1 of Figure 3.12 (B) and lane 7 of Figure 3.20 (C). (b) shows lane 21 of Figure 3.18 (X), lane 16 of Figure 3.20 (Y) and lane 8 of Figure 3.22 (Z). Lanes M: same marker, 1 Kb ladder.

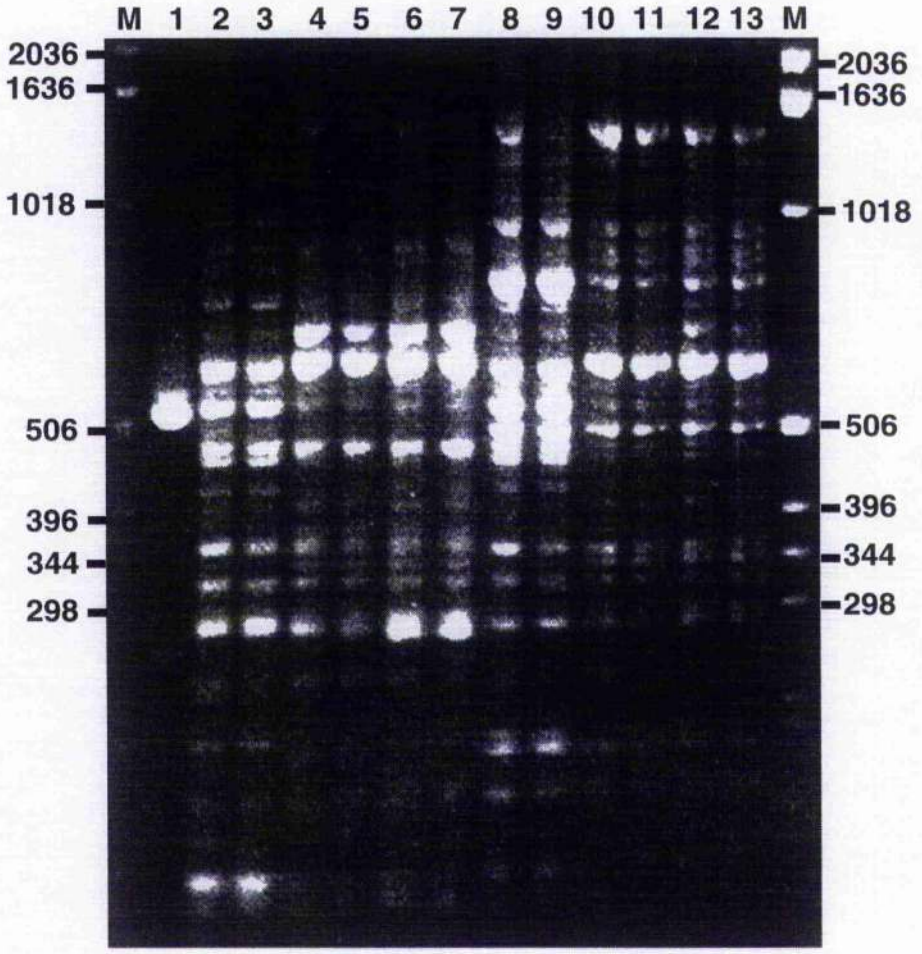

Figure 3.24 ***The effect of using different batches of REP primers on the PCR fingerprints of H. somnus strains THs and SA01.*** Lanes M: 1 Kb DNA Ladder. Lanes 1: positive control. Lanes 2-13: represents reactions 1-12 of **Table 2.7.**

expresses the ability to distinguish different strains. To simplify and standardise this expression, Hunter and Gaston (1988) proposed the use of Simpson's index of diversity derived from elementary probability theory. Simpson's index has been successfully applied to compare the typing efficency of *N. meningitidis* by MAb-based serotyping, MEE, ribotyping, PFGE and RFLP of PCR-ribotyping (Swaminathan *et al.*, 1996). Thus, Simpson's indices of diversity for each of the present methods, REP-PCR, ERIC-PCR and PCR-ribotyping were respectively 84, 92 and 76 for *H. somnus*, 88, 82 and 81 for *H. ovis* and 78, 79 and 8 for *A. seminis* (**Table 3.11**). The combined indices of diversity of these three methods were 90, 94 and 89 respectively for *H. somnus*, *H. ovis* and *A. seminis*. The combination of three methods was especially useful for the typing *A. seminis* as the PCR-ribotyping made little contribution to strain differentiation for these isolates. This clearly shows for *A. seminis* as the number of different types in each method was relatively lower than for *H. somnus* and *H. ovis*. The poor correlation of strains between types has increased this combined discrimination.

3.4 PLASMID PROFILES OF *H. SOMNUS*, *H. OVIS* AND *A. SEMINIS* ISOLATES

Four of the 29 *H. somnus* isolates (13%) contained single plasmids. These isolates were SA11, SA13, SA52 and X4 whose plasmids were 3.5, 1.7, 1.5 and 3.1 kb in size respectively (**Figure 3.25**). Twelve of the 19 *H. ovis* isolates (63%) showed the presence of plasmids and, among them, seven isolates (SA16, SA26, SA27, SA28, SA44, SA54, SA55) had similar profiles, containing two plasmids of 3.7 and 5.0 kb (**Figure 3.25**). Isolates SA72 and SA73 also showed two plasmids, of 2.3 and 2.9 kb in the former and 2.5 kb and 3.0 kb in the latter. Three isolates (SA29, SA45 and SA46) showed one plasmid of 2.9 kb. Among the *A. seminis*, two isolates (8%), SA62 and SA63, showed plasmids. Both contained two plasmids each and their sizes were 2.6 and 4.6 kb for SA62 and 3.8 and 4.9 kb for SA63. In general, the plasmids of these isolates were of low copy number when compared with *E. coli* containing pUC19 (**Figure 3.25**). For this reason, an attempt to characterise these plasmids by restriction mapping was unsuccessful as the yield of plasmid DNA of the isolates was not enough.

Antibiotic sensitivity pattern of plasmid bearing isolates. There was no significant difference in antibiotic sensitivity patterns between the plasmid-bearing and plasmidless isolates to a range of antibiotics tested. A similar pattern of antibiotic resistance for all isolates was observed (**Table 3.12**) except with cotrimoxazole where *H. somnus* and *H. ovis* were generally resistant whereas *A. seminis* was sensitive to this antibiotic.

Table 3.11 ***Discrimination indices of the PCR typing methods***[¶]

Species		*H. somnus*	*H. ovis*	*A. seminis*
	Number of isolates	29	19	24
REP-PCR	REP types	11	11	5
	Discrimination index (%)	**84**	**88**	**78**
ERIC-PCR	ERIC types	16	7	9
	Discrimination index (%)	**92**	**82**	**79**
PCR-	Ribo types	8	5	2
Ribotyping	**Discrimination index (%)**	**76**	**81**	**8**
Combined Discrimination index (%)		90	94	89

[¶] Calculated by the method of Hunter and Gaston (1988).

The equation for the calculation of the index is derived from elementary probability theory.

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j-1)$$

D - discrimination index

N - total number of strains in the sample population

s - total number of types described

n_j- the number of strains belongs to the jth type

Example: Discrimination index for REP-PCR typing of *H. somnus* isolates (**Table 3.5**).

N = 29, s = 11, $n_1 = 11$, $n_2 = 3$, $n_3 = 3$, $n_4 = 3$, $n_5 = 2$, $n_6 = 2$, $n_7 = 1$, $n_8 = 1$, $n_9 = 1$, $n_{10} = 1$ and $n_{11} = 1$.

D = 1- {[(11x10)+(3x2)+(3x2)+(3x2)+(2x1)+(2x1)+(1x0)+(1x0)
+(1x0)+(1x0)+(1x0)]/[29x28]}
= 1- {[110+6+6+6+2+2+0+0+0+0+0]/812]}
= 1- {132/812}
= 1- 0.16
D = 0.84
D% = 84

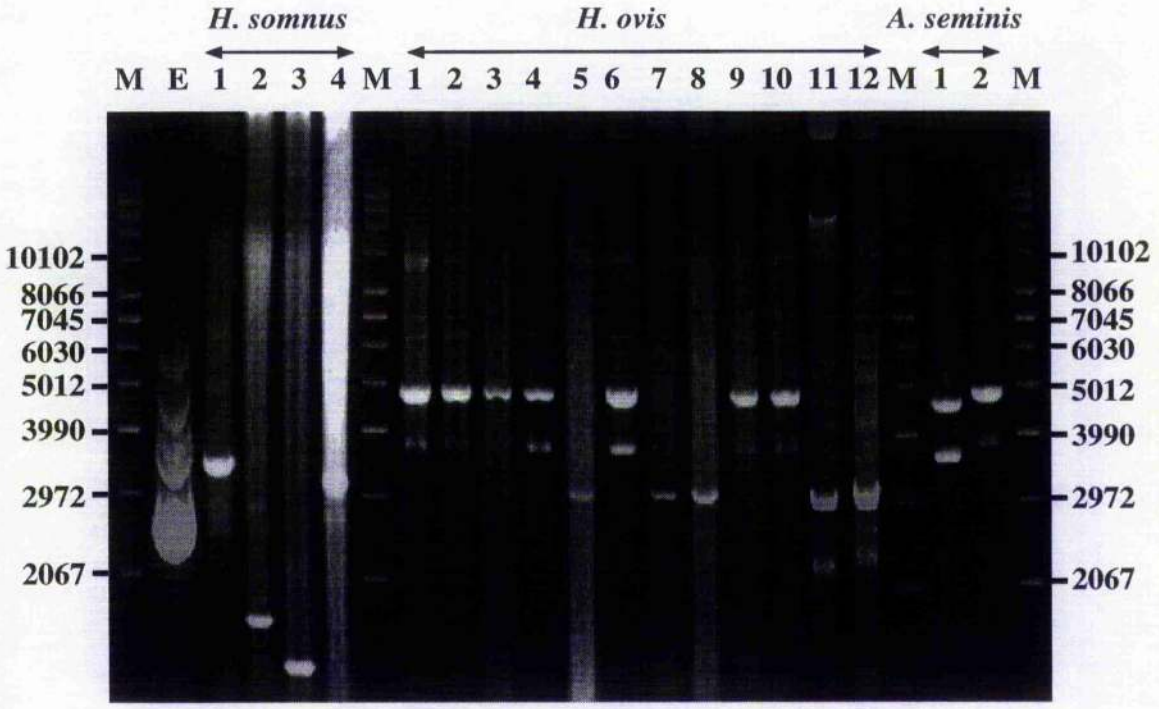

Figure 3.25 ***Plasmid profiles of H. somnus, H. ovis and A. seminis isolates.*** M-Marker, Supercoiled DNA Ladder. E- Control, *E. coli* pUC19. *H. somnus* lanes 1-4: SA11, SA13, SA52 and X4 respectively. *H. ovis* lanes 1-12: SA16, SA26, SA27, SA28, SA29, SA44, SA45, SA46, SA54, SA55, SA72 and SA73 respectively. *A. seminis* lanes 1-2: SA62 and SA63 respectively.

Table 3.12 ***Antibiotic sensitivity pattern of plasmid containing isolates*** ‡

Isolate§	Antibiotic§							
	PG	AP	AUG	S	T	C	TS	ENR
H. somnus SA11	29	33	29	10	22	27	0	25
H. somnus SA13	26	31	27	14	23	28	12	26
H. somnus SA52	31	33	31	13	21	31	0	28
H. somnus X4	32	32	33	13	21	31	11	29
H. somnus THs*	26	26	28	11	18	29	0	27
H. ovis SA16	31	32	32	11	20	29	0	25
H. ovis SA26	32	33	35	10	20	27	0	26
H. ovis SA27	32	35	36	10	19	28	0	30
H. ovis SA28	41	38	35	13	21	36	0	30
H. ovis SA29	27	32	29	10	21	31	0	30
H. ovis SA44	30	24	33	8	21	29	14	29
H. ovis SA45	31	33	27	10	19	33	0	32
H. ovis SA46	28	23	29	9	20	31	16	30
H. ovis SA54	36	35	35	13	21	29	0	30
H. ovis SA55	33	32	34	14	19	29	0	28
H. ovis SA71	31	35	34	11	21	30	0	30
H. ovis SA72	33	29	30	9	21	29	0	27
H. ovis SA24*	29	31	28	11	17	26	0	27
A. seminis SA62	27	30	28	10	28	33	32	26
A. seminis SA63	32	30	30	15	24	29	29	26
A. seminis TAs*	28	30	30	12	22	30	18	27

‡ The diameter of the inhibitory zones is given in mm.

§ Plasmid bearing isolates: *H. somnus* SA11, SA13, SA52 and X4; *H. ovis* SA16, SA26, SA27, SA28, SA29, SA44, SA45, SA46, SA54, SA55, SA71 and SA72; *A. seminis* SA62 and SA63. Plasmid-less isolates *H. somnus* THs, *H. ovis* SA24 and *A. seminis* TAs included as controls.

§ Antibioitics used and their strength: PG, penicillin G 10 units; AP, ampicillin 10 µg; AUG, augmentin 30 µg; S, streptomycin 25 µg; T, tetracycline 10 µg; C, chloramphenicol 10 µg; TS, cotrimoxazole 25 µg; ENR, enrofloxacin 5 µg.

* Plasmidless isolates as controls.

3.5 DEVELOPMENT OF *A. SEMINIS*-SPECIFIC PRIMERS

3.5.1 Sequences of PCR-ribotyping products of *A. seminis*

The PCR-ribotyping products were successfully cloned with a view to manual sequencing and the inserts were confirmed by double restriction digestion (**section 2.8.1.6**) as shown in **Figure 3.26**. Several attempts of manual sequencing were not fruitful as the data did not yield more than 200 nucleotides including the vector sequences. Later, a centralised automated sequencing service was available and the facility was successfully used for sequencing the cloned PCR products. PCR-ribotyping with primers GIRRN and LIRRN produced similar amplimers from all *A. seminis* isolates (**Figure 3.18**). The sequences of these amplimers from the *A. seminis* type strain ATCC #43626 showed that those two bands are 562 bp and 691 bp in size. Both sequences contained the 16S rRNA partial sequence, a spacer region and the 23S rRNA partial sequence along with the PCR-ribotyping GIRRN and LIRRN primers at the ends (**Figures 3.27 a** and **b**).

3.5.2 rRNA operons of *A. seminis*

The *A. seminis* genome contains at least two RNA operons as the spacer regions were clearly different as shown in **Figures 3.27 a** and **b**. The regions of the RNA operons were defined by comparing the sequences with those in the Genbank database and by use of the guidelines suggested by Gurtler and Stanisich (1996). The smaller PCR amplimer, ribosomal operon *rrnA*, consisted of a part of the 16S RNA gene, a tRNA gene for glutamine and part of the 23S RNA gene. The larger PCR amplimer, ribosomal operon *rrnB*, contained part of the 16S RNA sequence, two tRNA genes, for isoleucine and alanine, and part of the 23S RNA sequence (**Figure 3.28**).

3.5.3 Sequence similarity to other bacteria

The sequences in **Figure 3.27 a** and **b** showed partial similarity to most of the Gram-negative and Gram-positive bacterial sequences in the Genbank database. The similarity was confined mainly to the 16S and 23S RNA and tRNA genes. The highest similarity was observed with those of *Haemophilus influenzae* where the *A. seminis* 16S rRNA, tRNA-Glu and tRNA-Ile sequences in **Figure 3.27** were identical and the 23S rRNA and tRNA-Ala sequences showed 97% similarity with those of *H. influenzae*.

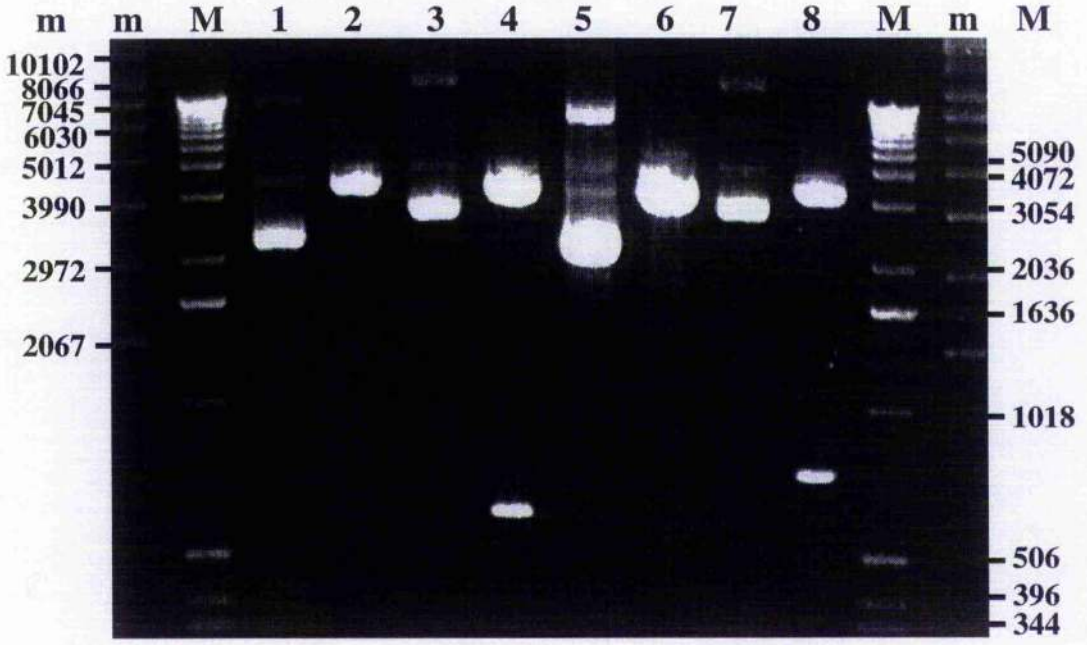

Figure 3.26 ***Restriction analysis with SacI and EcoRI of double-digested plasmid DNA showing the vector and inserts.*** Lanes m and M: Supercoiled DNA Ladder and 1 Kb DNA Ladder, respectively. Lanes 1-4 deal with insert of short PCR product; 1: plasmid vector - no insert, 2: double digested plasmid vector- no insert, 3: plasmid vector + insert, 4: double digested plasmid vector + insert. Lanes 5-8 deal with insert of large PCR product; 5: plasmid vector - no insert, 6: double digested plasmid vector - no insert, 7: plasmid vector + insert and 8: double digested plasmid vector+ insert.

Figure 3.27a *562 bp amplimer (rrnA)*

```
   1 GAAGTCGTAA CAAGGTAACC GTAGGGGAAC CTGCGGTTGG ATCACCTCCT
  50 TAACTGAATG AAGTGATAGC GAGTGTTCAC ACAGATTGGC TGAGATATTG
 100 TAGACAGAAA AGAATAAGAG AAGAGACCGC ACTTTTAGAT TGCTTACTAG
 150 GTCGAGTAAT AGAAGTCGTA TTAATAAAGA TTAATATCAA TATCAAGGAT
 200 GAAAGTGCGA GGAAAGCAAA GAGATTGTCT TTACCTGATG TCCCCATCGT
 250 CTAGAGGCCT AGGACATCGC CCTTTCACGG CGGTAACCGG GGTTCGAATC
 300 CCCGTGGGGA CGCCAATTAA AGATGATTTT AAATTATCTT ATTGTTCTTT
 350 AAAAAATAGG AAACAAGCTG AAAACTGAGA GATTTTTCAA GTCAGGGCTT
 400 AAGAAAGATA AGCGCTGAGA GAAGGAAAGT CTGAGTAGTT CGAAAGAAAG
 450 AAATCTTAAC TGAAGAAAGG CGGTTAAGTG TTTAGTTGAA AGATATCGCC
 500 TTAAGCATAA AATGCTTGAG GTTGTATGGT TAAGTGACTA AGCGTACACG
 550 GTGGATGCCT TG
```

Figure 3.27b *691 bp amplimer (rrnB)*

```
   1 GAAGTCGTAA CAAGGTAACC GTAGGGGAAC CTGCGGTTGG ATCACCTCCT
  50 TAACTGAATG AAGTGATAGC GAGTGTTCAC ACAGATTGGC TGAGATATTG
 100 TAGACAAGAA AAAGACGAAG AGACATTCTT TTGGGTCTGT AGCTCAGGTG
 150 GTTAGAGCGC ACCCCTGATA AGGGTGAGGT CGGTGGTTCA AGTCCACTCA
 200 GACCCACCAC TCAATGCTAA TATAGCATAC TTACAGAAAG TACAAAGTAA
 250 TATAAATTGA GTGAGAGTGA AAGCTAAAGC GAAGTTGATG ACTGGGGATA
 300 TAGCTTAGCT GGGAGAGCGC CTGCCTTGCA CGCAGGAGGT CAGCGGTTCC
 350 ATCCCGCTTA TCTCCACCAA ATCATCATGC ACTAAGTGCA TAGTGTAAAC
 400 CCACTTTATG GGTGATGAAT TTTATTAATA TGTATTTAGT CATGATGATT
 450 TGCCGAAAGG CAAATGTCTA TTGTTCTTTA AAAAATAGGA AACAAGCTGA
 500 AAACTGAGAG ATTTTTCAAG TCAGGGCTTA AGAAAGATAA GCGCTGAGAG
 550 AAGGAAAGTC TGAGTAGTTC GAAAGAAAGA AATCTTAACT GAAGAAAGGC
 600 GGTTAAGTGT TTAGTTGAAA GATATCGCCT TAAGCATAAA ATGCTTGAGG
 650 TTGTATGGTT AAGTGACTAA GCGTACACGG TGGATGCCTT G
```

Figure 3.27 ***The sequences of the two amplification products from PCR-ribotyping of A. seminis.*** (a) Sequence of the smaller spacer region. Bold sequences: 1-52, 16S rRNA partial sequence; 240-308, tRNA-Glu; 538-562, 23S rRNA partial sequence. (b) Sequence of the larger spacer region. Bold sequences: 1-52, 16S rRNA partial sequence; 133-209, tRNA-Ile; 294-370, tRNA-Ala; 667-691, 23S rRNA partial sequence. Boxed sequences are primers SRJAS2, SRJAS3 and SRJAS1 respectively. PCR-ribotyping primer sequences are underlined.

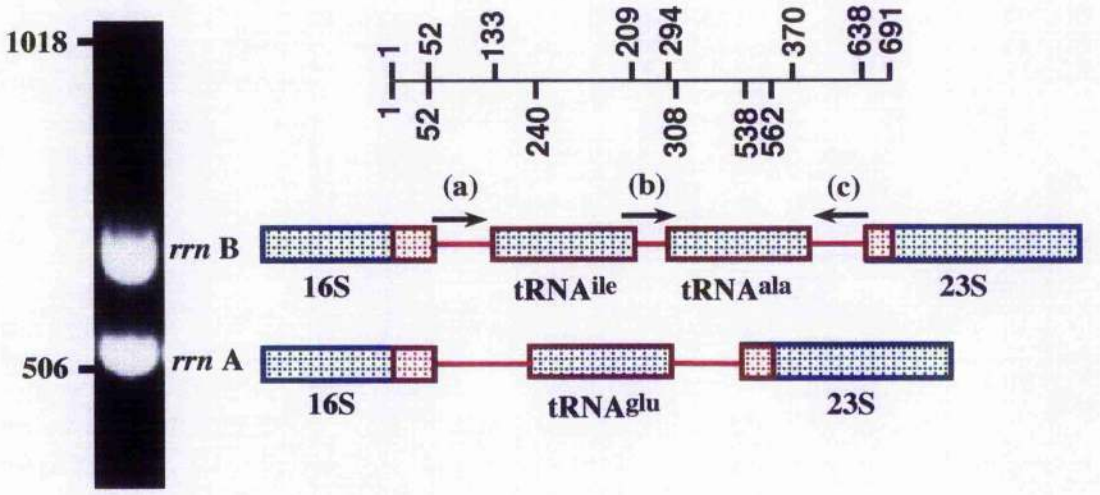

Figure 3.28 ***Structure of the spacer region of two RNA operons of A. seminis.*** The two amplification products of PCR-ribotyping are shown on the left. The arrows a and b denote forward primers SRJAS2 and SRJAS3 respectively and arrow c denotes the reverse primer SRJAS1. The numbers above the spacer regions represent base number in the two sequences.

3.5.4 Design of *A. seminis*-specific primers

There were two regions of the larger spacer sequence that did not match with any sequence in the Genbank database and those two sequences were used to design primers SRJAS1 and SRJAS3 (**Figure 3.27b**). PCR amplification of DNA templates from different *A. seminis* strains produced multiple bands with this primer combination instead of a single amplimer as expected (data not shown). To avoid this, a new primer, SRJAS2, was designed from another region of the larger sequence (**Figure 3.27b**) where there was no matching with the other bacterial sequences deposited in the Genbank. PCR of *A. seminis* DNA with the primer combination of SRJAS1 and SRJAS2 amplified only the expected 436 bp band from the larger spacer region *rrnB*. The species-specific PCR assay for *A. seminis* was optimised as described (**Table 2.5** and **2.6**) and the optimised levels were 0.2 mM dNTPs, 3 mM $MgCl_2$, 50 pM of each primer and 2.5 µl of template preparation, as shown in lane 2 of **Figure 3.29**. These primers yielded a 436 bp amplimer from all of the 24 *A. seminis* isolates tested. **Figure 3.30** shows the results for representative isolates of *A. seminis*. These primers did not show any amplification products when boiled cell extracts of other bacterial isolates, including *H. somnus* and *H. ovis*, were used as template DNA (**Figure 3.30** and **Figure 3.31**).

3.5.5 Sensitivity of the PCR assay

One of the potential applications of PCR methods which take advantage of its high sensitivity is in the detection of specific pathogens in clinical samples. The PCR with the above *A. seminis*-specific primers was assessed for its ability to detect low numbers of *A. seminis* in ram semen samples. First, for preliminary sensitivity and detection experiments, the sample volume in the usual PCR reaction was increased from 2.5 µl to 10 µl in order to increase the probability that target DNA template was present in the reaction. The detection limit with primers SRJAS1 and SRJAS2 for *A. seminis* diluted in distilled water was approximately six CFU per 10 µl sample when 5% (w/v) Chelex 100 was included in the sample preparation. The sensitivity was four-fold lower in the absence of Chelex 100 (data not shown). Therefore, Chelex 100 was included in all subsequent sample preparations. Due to problems with viscosity, even with semen in storage solution (one part of semen: three parts storage solution), a preliminary 10-fold dilution of semen specimen was required prior to sample preparation. When *A. seminis* was added to 1000-fold diluted ram semen without storage solution in distilled water, the detection limit was again approximately six CFU per 10 µl sample and if the semen was diluted 1:10 or 1:100, there

Figure 3.29 ***Optimisation of PCR mixture for A. seminis-specific primers***. Lanes M: 1 Kb DNA Ladder. Lanes C and 1-10 correspond to the reactions (control and 1-10) outlined in **Table 2.5** respectively.

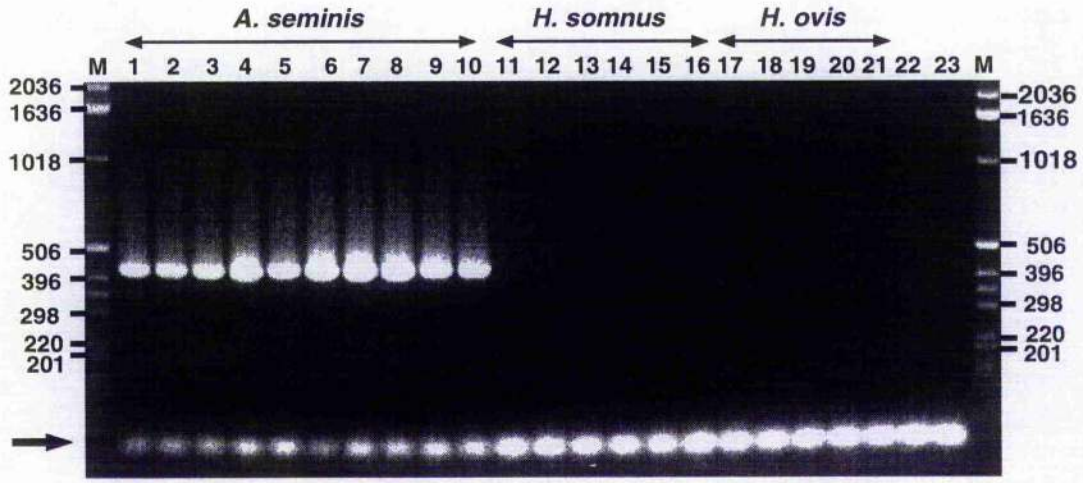

Figure 3.30 ***Specificity of the PCR assay 1.*** Lanes M: 1 Kb Ladder. Lanes 1-10: *A. seminis* isolates, TAs, SA25, SA30, SA33, SA35, SA37, SA43, SA60, SA63, SA67 and SA70. Lanes 11-16: *H. somnus* isolates THs, SA01, SA04, SA07, SA12 and SA20. Lanes 17-21: *H. ovis* isolates SA08, SA16, SA26, SA53 and SA69. Lanes 22-23: negative controls. The arrow indicates the presence of primer dimers.

Figure 3.31 ***Specificity of the PCR assay 2.*** Lanes M: 1 Kb Ladder. Lanes 1-2: *A. seminis* strains TAs and SA33. Lanes 3: *A. actinomycetemcomitans*, 4: *E. coli* K12, 5: representative isolate of *H. somnus*, 6: representative isolate of *H. ovis*, 7: representative isolate of *P. multocida* D, 8: representative isolate of *P. multocida* B2, 9-10: two isolates of *P. haemolytica*, 11: *P. trehalosi*, 12-17: "unknown" isolates from the bovine reproductive tract, lanes 18-20: unknown isolates from the ovine reproductive tract, 21-22: negative controls. The arrow indicates the presence of primer dimers.

was no obvious difference in sensitivity levels (**Figure 3.33**, lanes 13-20). In a separate experiment, the sensitivity level was approximately three CFU per 10 µl sample with 100-fold diluted semen without storage solution (**Figure 3.32**, lanes 1-5). The semen storage solution was found to be inhibitory to the PCR reaction because with 100-fold dilution of semen in storage solution the detection limit was 320 CFU per 10 µl reaction sample (**Figure 3.32**, lanes 6-10). Proteinase K treatment of semen in storage solution increased the sensitivity 100-fold i.e. detection of approximately three CFU per 10 µl sample (**Figure 3.32**, lanes 16-20). There was no difference in sensitivity when the semen samples in the absence of storage solution were treated with proteinase K prior to boiling (**Figure 3.32**, lanes 11-15). This showed that the storage solution, but not the components in semen itself, inhibited the PCR when semen was diluted 100-fold. This was comfirmed in a separate experiment, in which seminal plasma, prepared by filtration of a 10-fold diluted semen specimen through a 0.22 µm filter (Millipore), did not show any inhibitory effect on the PCR reaction. Storage solution with egg yolk was 100-fold more inhibitory than storage solution prepared in the absence of egg yolk (data not shown).

3.5.6 Detection of *A. seminis* from naturally contaminated semen

Naturally infected ram semen specimens, which had been stored frozen for various lengths of time, and from which *A. seminis* had previously been isolated and identified by biochemical phenotyping and by API ZYM, were also tested. Six semen samples were assessed for the level of *A. seminis* contamination initially by plate counts. They were: two heavily contaminated specimens (both contaminated between 7 and 8 x 10^7 CFU/ml); a low contaminated specimen (150 CFU/ml) without storage solution; and three semen samples in storage solution (containing *A. seminis* at 40-180 CFU/ml). These semen samples were diluted 1/10 and 1/100 and subjected to PCR with primers SRJAS1 and SRJAS2 after treatment with proteinase K and boiling in the presence of Chelex 100. With the heavily contaminated semen sample, *A. seminis* was detectable, as shown by a clear 436 bp amplimer (**Figure 3.33**). However, no PCR products were obtained from the specimens contaminated with low numbers of *A. seminis*. This indicated that the lower limit of detection of *A. seminis* in semen samples was 300 CFU/ml as a positive reaction was obtained at this bacterial concentration (**Figure 3.33**).

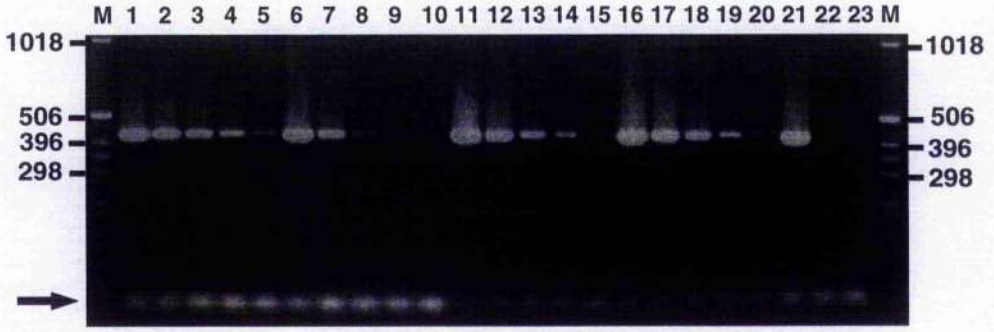

Figure 3.32 ***Detection of A. seminis in artificially-contaminated ram semen by PCR.*** DNA samples were prepared by boiling with 5% (w/v) Chelex 100. All samples contained a 1:100 dilution of semen with and without storage solution plus added 10-fold serial dilutions of *A. seminis*. Lanes M: 1 Kb Ladder. Lanes 1-5: semen without storage solution, 3 x 10^4, 3 x 10^3, 3 x 10^2, 30, three CFU per 10 µl sample respectively. Lanes 6-10: semen with storage solution and the same CFU/sample as lanes 1-5. Lanes 11-15: semen without storage solution but with proteinase K treatment for template preparation and the same CFU/sample as lanes 1-5. Lanes 16-20: semen with storage solution and with proteinase K treatment and the same CFU/sample as lanes 1-5. Lane 21: positive control; *A. seminis* at the dilution of lane 1 in distilled water. Lanes 22 and 23: negative controls. At the final dilutions used in lanes 5, 10, 15 and 20, 320 CFU/ml of *A. seminis* was detected by plate counting, equivalent to approximately three CFU per 10 µl sample. The arrow indicates the presence of primer dimers.

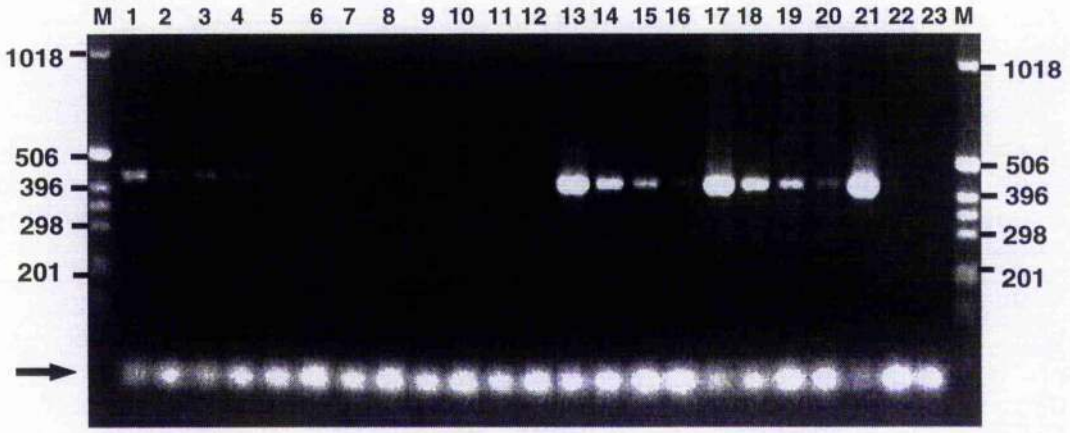

Figure 3.33 ***Detection of A. seminis from naturally-infected ram semen by PCR.*** Samples at the specified dilutions were treated with proteinase K and the template DNA was prepared by boiling with 5% (w/v) Chelex 100. Lanes M: 1 Kb Ladder. Lanes 1-12: six *A. seminis* contaminated ram semen samples. The odd numbered lanes were 1:10 dilutions and the even numbered lanes were 1:100 dilutions of the semen samples. Lanes 1-2 and 3-4, samples of heavily contaminated semen and lanes 5-6, low contaminated semen. These samples had no added storage solution. Lanes 7-12, low contaminated semen with added storage solution. Lanes 13-16, 10-fold dilutions of *A. seminis* in 10-fold diluted semen with no added storage solution, 5 x 10^3, 5 x 10^2, 50 and 5 CFU per 10 µl sample respectively. Lanes 17-20, 100-fold diluted semen and the same *A. seminis* dilutions as in lanes 13-16. Lane 21, positive control (5 x 10^3 CFU of *A. seminis*/sample in distilled water). Lanes 22-23, negative controls. The arrow indicates the presence of primer dimers.

3.5.7 Attempt to detect *A. seminis* from tissue samples

Attempts to detect *A. seminis* from any of the tissue samples even after processing with proteinase K lysis buffer and Chelex 100 were unsuccessful. The 1 in 10 dilution of tissue samples were found to be inhibitory for PCR as shown by lane 1 of the controls in **Figure 3.34** representing tissue sample to which was added approximately 6.4 x 10^5 *A. seminis*. There was no amplification of the target sequence and the lane 2 band (1/100 dilution of tissue) was less intense than bands in lanes 3 and 4 representing further diluted samples.

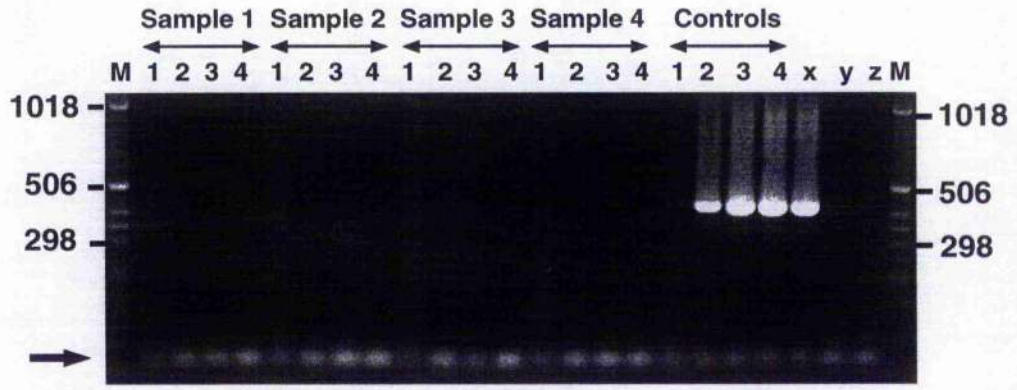

Figure 3.34 ***Attempt to detect A. seminis from tissue samples.*** Lanes 1-4 of samples 1-4: 10-fold dilutions of homogenised tissue extract. Lanes 1-4 of controls: lanes 1-4 of tissue sample 1 with equal amount of *A. seminis* cells. Lane x: positive control and lanes y and z: negative controls. The arrow indicates the presence of primer dimers.

4. DISCUSSION

4.1 THE ISOLATES

Haemophilus somnus. The type strain of *H. somnus* (designated as THs for this study) was from the American Type Culture Collection (ATCC). Unfortunately, the origin of this isolate was not traceable except that it originated from Wisconsin State Animal Health Laboratory, USA. The only record held at the ATCC was that it had been deposited by Professor C. J. Czuprynski (Czuprynski and Hamilton, 1985a). The field isolates were representative of all parts of Scotland. Half of the *H. somnus* isolates were respiratory isolates of which the majority were from pneumonic cases and many of the reproductive isolates were from cases with reproductive problems (**Table 2.1**).

Histophilus ovis. The reference strain, designated in this study as SA24, was from a case of ovine epididymitis and was the first reported isolate of *H. ovis* in the UK (Low and Graham, 1985). Although *H. ovis* causes various disease conditions in sheep, all of these isolates were of reproductive tract origin, from subfertile and infertile cases and from normal animals. The isolates were representative of almost all parts of Scotland (**Table 2.2**).

Actinobacillus seminis. The type strain of *A. seminis*, designated here as TAs, was from the National Collection of Type Cultures (NCTC). This was the first reported isolate of *A. seminis*, from a case of ovine epididymitis in Australia (Baynes and Simmons, 1960). *A. seminis* has been reported as specific for sheep (Phillips, 1984) but there are reports of its isolation from cattle (Dixon *et al.*, 1983). All isolates employed in this study were from sheep except one that was isolated from the reproductive tract of a cow (**section 2.5**). All sheep isolates were from semen except strain SA36 which was from a preputial washing. These isolates were from both clinically normal animals as well as from diseased animals from different areas of Scotland.

4.2 BIOCHEMICAL PROPERTIES OF THE ISOLATES

Conventional biochemical properties. The purpose of detemination of the biochemical activities of these isolates was to confirm their identity before their use in other experiments. These tests were chosen on the basis of previous work where they were reported to give clear positive or negative results for the species (Kennedy *et al.*, 1960; Stephens *et al.*, 1983; Hajtos *et al.*, 1987). With the biochemical tests used, the *H. somnus* isolates were indistinguishable from the *H. ovis* isolates, in agreement with previous reports (Kennedy *et al.*, 1960; Stephens *et al.*, 1983). The differentiation of *H. somnus* from *H. ovis* was essentially the host i.e. the isolates from cattle with the properties

described by Kennedy *et al.* (1960) and Stephens *et al.* (1983) were called *H. somnus* and similar isolates from sheep were called *H. ovis*. The colonies of 48 h cultures of these three species on BHIBYE were indistinguishable from each other.

Only two biochemical tests, namely catalase and indole production were able to differentiate *A. seminis* from *H. somnus* and *H. ovis*. The sheep isolates of *A. seminis* showed results comparable with those reported for *A. seminis* isolates from sheep by Hajtos *et al.* (1987) except for glucose and xylose. The *A. seminis* isolates in this study were negative for fermentation of glucose, except two, but all *A. seminis* isolates (n=18) in their study were positive. The *A. seminis* isolates in this study showed variable results (majority positive of which some of them were weak reactions or negative) for xylose fermentation. All isolates reported by Hajtos *et al.* (1987) were positive for xylose fermentation. These minor variations may be due to differences of methodology. The bovine isolate of *A. seminis* (X16) gave identical results with those of Hajtos *et al.* (1987).

The first identifications of the three species were made on the basis of cultural and biochemical properties (Roberts, 1956; Baynes and Simmons, 1960; Kennedy *et al.*, 1960). Although their taxonomic status is not clear, *H. somnus* and *H. ovis* have been included in the *Haemophilus-Histophilus* group on the basis of their similar morphological, biochemical, antigenic and cytochemical properties with *Haemophilus agni*. Kirkham *et al.* (1989) concluded that biochemical characteristics were of no use in differentiating members of this H-H group. However, biochemical properties have been used to differentiate isolates of *H. somnus* and *H. ovis*. There were 21 different biotypes identified among 105 *H. somnus* isolates in a study by Fussing and Wegener (1993). Ward *et al.* (1995) showed three biotypes for six *H. somnus* isolates and three biotypes for 12 *H. ovis* isolates. These observations were based on 21 tests and the variability was seen only with five of 21 tests. In general, these species are fastidious, nutritionally demanding organisms. Many of the biochemical tests depend on growth on a particular test medium and depend on the quality of the test medium and on incubation conditions. In general, the biochemical methods are labour intensive and time consuming.

The identification of *A. seminis* has also been based on cultural and biochemical properties of the organism. It was excluded from the H-H group on the grounds of its lack of yellow pigment, production of catalase and differences in cell envelope protein profiles (Stephens *et al.*, 1983). Hajtos *et al.* (1987) reported that *A. seminis* was biochemically active in suitable media and noted that the slight variation in biochemical properties obtained for different strains by different authors may be in part due to the occurrence of different biotypes. However, there were no subsequent reports to support this biovariability among *A. seminis* isolates.

Usefulness of API ZYM method. The API ZYM system has also been used for the identification of *H. somnus* and related organisms (Groom *et al.*, 1986), but it is not a reliable method to differentiate *H. somnus* from *H. ovis*. The API ZYM system was used in this study as a supplementary method for the confirmation of the identity of the isolates. The results obtained for the three species were very similar to the findings of Groom *et al.* (1986), except that none of the present *H. ovis* isolates showed α-fucosidase activity, while some of *H. somnus* isolates gave a positive reaction for this test. In contrast, none of *H. somnus* isolates of Cousins and Lloyd (1988) were positive whereas some of the *H. ovis* isolates were positive in this test.

A. seminis isolates were differentiated from the other two species as they give a strong colour reactions for API ZYM, and by simple conventional methods such as catalase test (Cousins and Lloyd, 1988). The API ZYM system has become the routine method for confirmation of the identity of *A. seminis* (Cousins and Lloyd, 1988; Low *et al.*, 1995) and the API ZYM profiles for the isolates examined in the present study agree with the previous results (Cousins and Lloyd, 1988). High intensity reactions for leucine arylamidase, acid phosphatase and β-glucuronidase, with little variation of profiles between strains, are characteristic features of *A. seminis*. Thus, the API ZYM system is useful for identification of *A. seminis* but is of little value in strain differentiation.

4.3 ISOLATION OF *H. SOMNUS* AND *A. SEMINIS* FROM SLAUGHTERHOUSE SPECIMENS

Available selective media for isolation of *H. somnus*. The selective medium for *H. somnus* used in this study (Slee and Stephens, 1985), did not completely suppress the growth of all other bacteria but it reduced the number and types of other bacteria sufficiently to allow recognition and isolation of *H. somnus*-like colonies. Ward *et al.* (1986) showed that this selective medium was better than the selective medium originally described by Ward *et al.* (1983) and the non-selective medium of BHIBYE with 5% (v/v) bovine blood, for isolation of *H. somnus* from nasal, vaginal and cervical samples. The use of this selective medium along with a non-selective medium was recommended, as some isolates from reproductive tract samples did not grow on the selective medium. However, this may have been due to the change of source of blood from equine to bovine by Ward *et al.* (1986). Kwiecian and Little (1989) reported that this selective medium gave a lower isolation rate of *H. somnus* than on a non-selective medium, but Slee and Stephens (1989) replied that this failure was due to two reasons: first the medium was not duplicated correctly and second that a difference in CO_2 percentage in the incubator would change the pH of the medium. Thus, the requirement for a better selective

medium still exists as none of these selective media are totally efficient in isolating *H. somnus* from clinical materials.

Prevalence of *H. somnus* in the bovine reproductive tract. Using the selective medium of Slee and Stephens (1985) *H. somnus* was isolated from four of 22 (18%) bovine reproductive tracts examined. Cervices of all four tracts contained *H. somnus* which was present in the vestibular opening of three of these tracts. Three vaginas and two uteri were also positive for the presence of *H. somnus*. The presence of mucopurulent discharge in three of these four reproductive tracts indicated the pathogenic involvement of these isolates. Previously a poor correlation between the isolation of *H. somnus* and the presence of inflammatory lesions had been reported (Miller *et al.*, 1983b). The extent of virulence of these isolates is difficult to determine due to lack of a small animal model for virulence tests. The vestibular gland has been reported as the reservoir for *H. somnus* as three of 24 major vestibular glands yielded *H. somnus* (Miller *et al.*, 1983a). Eight of 100 female reproductive tracts showed the presence of *H. somnus*, and of those, seven vaginas, two uteri, two cervices and a urine sample yielded this organism. In a separate slaughterhouse survey, there was a 6.1% isolation rate for *H. somnus* in reproductive tract of cows (Kwiecien and Little, 1992). Isolates were recovered from both normal and diseased reproductive tracts. The occurrence of pathogenic isolates was demonstrated by intracisternal inoculation of young calves. The sites of isolation were not reported and the relationship of clinical status to the isolation of the organism was not evident even in this study (Kwiecien and Little, 1992). The clinical involvement of the other contaminating bacteria encounterd in this survey was not known.

The bovine strain X16 was isolated from the vestibular opening of a cow during a study of *H. somnus* isolates collected from slaughterhouse materials. This isolate was culturally indistinguishable from *H. somnus* but was catalase positive. There has been a previous report of *A. seminis* being isolated from cattle (Dixon *et al.*, 1983), but those isolates were catalase negative. Because *A. seminis* is a catalase positive organism (Hajtos *et al.*, 1987; Sneath and Stevens, 1990) the identity of the isolates in that report was not clear. They seem to be *H. somnus* but the reason for calling them *A. seminis* was not clear. The lack of evidence for the presence of *H. somnus* in Australia may be a reason. Although, there is a possibility of cross contamination of bovine and ovine carcasses in the slaughterhouse, isolate X16 studied here was catalase positive and API ZYM confirmed that it was an *A. seminis* isolate. The REP and ERIC fingerprints of this isolate showed markers common to other *A. seminis* isolates and it showed an identical pattern for PCR-ribotyping with that of other *A. seminis* isolates (**Table 3.10**). This is the first reported isolation of a "typical" *A. seminis* strain from cattle and this isolate may well represent a subtype of *A. seminis* of bovine origin. Screening of a large number of bovine samples

could help to clarify this point. The lack of a suitable selective medium may hamper these attempts and development of a selective medium for the isolation of *A. seminis* would therefore be useful.

4.4 FINGERPRINTING OF ISOLATES BY PCR

PCR is a very powerful technique but slight variations in its components may adversely affect its performance. For this reason, optimisation of the components and reaction conditions is necessary to avoid the problems with reproducibility. A simple procedure for the optimisation of PCR, based on orthogonal arrays, developed by Cobb and Clarkson (1994) proved to be highly useful. It reduced not only the time but also the cost and labour involved in trying out numerous different permutations of reaction conditions. The optimisation studies showed that the optimum concentrations of dNTPs, $MgCl_2$, primer and template varied with the three species but not with the type of PCR (**Table 3.4**). The optimum annealing temperature also varied with the species. Extension time was optimal at six min but this is longer than usual for PCR (approximately 1kb/min). This may be an inherent character of these multi-amplicon PCR methods with a large size range of different products, a problem not present with single amplicon detection (J. Versalovic, personal communication).

Very recently, QIAGEN (QIAGEN Ltd., West Sussex, UK) has introduced a PCR kit which can be used without having to go through this optimisation process. They have included NH_4^+ in addition to K^+ in their PCR reaction buffer. The function of K^+ is to bind to the phosphate groups on the DNA structure and to stabilise the annealing of the primers to the template. The incorporated NH_4^+ is present both as ammonium ions and as ammonia under thermocycling conditions and can interact with the hydrogen bonds between the bases destabilising principally the weak hydrogen bonds at mismatched bases. The combined effect of these cations (K^+ and NH_4^+) maintains the high ratio of specific to non-specific primer-template binding over a wide temperature range (Loffert *et al.*, 1997). Incorporation of NH_4^+ also suppress the potentially marked effect that changes in Mg^{2+} concentration can have on PCR. In further studies, it would be of interest to compare results with this new PCR reaction buffer with those obtained with the present "optimised" system.

The REP and ERIC sequences were first described in *E. coli* and *Salmonella typhimurium* but they are widespread in all bacteria (Stern *et al.*, 1984; Hulton *et al.*, 1991; Versalovic *et al.*, 1991). The REP-PCR and ERIC-PCR typing methods based on these sequences have been used with many bacterial (Versalovic *et al.*, 1991) and fungal species

(van Belkum *et al.*, 1993). In the field of medical microbiology, these methods have been applied on numerous occasions. For example, *Legionella* species and strains have been differentiated according to their epidemiological origin by Georghiou *et al.* (1994) and the fingerprints were relatively easy to interpret compared with ribotyping, pulsed-field gel electrophoresis or restriction fragment length polymorphism analysis. Giesendorf *et al.* (1994) applied this REP- and ERIC-PCR to show differences between *Campylobacter* strains of different origins. These methods were particularly useful as other, conventional typing methods could not be applied to all *Campylobacter* strains and some isolates were untypable by these methods. *Enterobacter aerogenes* is a common cause of nosocomial infections and REP- and ERIC-PCR techniques have been used for the identification of hospital outbreak strains by Georghiou *et al.* (1995). They reported that REP-PCR was more discriminatory and produced less complex patterns than ERIC-PCR. In a comparative study of these two techniques and other molecular methods i.e. RAPD, restriction analysis of the amplified 16S rRNA gene and 16S-23S rRNA spacer region, with the *Acinetobacter calcoaceticus-A.baumannii* complex, REP-PCR showed the highest discriminatory index of 0.99, followed by ERIC-PCR with a discriminatory index of 0.94 (Vila *et al.*, 1996). The RAPD method showed a discriminatory index of 0.87 and the other two techniques were poorer than these.

In the veterinary field, these techniques have been applied widely. For example, REP- and ERIC-PCR have been applied to the identification of *E. coli* strains from bovine clinical mastitis (Lipman *et al.*, 1995). ERIC-PCR was recommended for epidemiological studies of *E. coli* mastitis, but REP-PCR was condemned on the grounds of poor reproducibility (discussed later). In a subsequent study, Lipman *et al.*, (1996) used ERIC-PCR, RAPD and phage typing for identification and subtyping of *Staphylococcus aureus* isolates from the bovine mammary gland. Both PCR techniques were useful for differentiation of *S. aureus* isolates and the phage typing did not give any additional information. On the other hand, Tcherneva *et al.* (1996) showed that REP-PCR was more highly discriminatory than ERIC-PCR for *Brucella* species and strains. REP-PCR provided a discriminatory method between strains in individual species of *Brucella* but ERIC-PCR only differentiated between species.

In the study of food borne pathogens, REP- and ERIC-PCR have been used to type species and strains of the genus *Listeria*. Both methods gave a similar degree of discrimination for all serotypes of *L. monocytogenes* except that REP-PCR showed a better discrimination among strains of serotype 1/2a (Jersek *et al.*, 1996). Toxigenic and non-toxigenic strains of *Vibrio cholerae* were clearly differentiated by REP-PCR and a substantial similarity among the REP-PCR fingerprints of many toxigenic *V. cholerae*

isolates suggested that a limited number of genotypes exists within the toxigenic group (Shangkuan *et al.*, 1997).

The presence of REP and ERIC repeat sequences in the *H. somnus*, *H. ovis* and *A. seminis* genomes and their usefulness for fingerprinting of these three species by REP- and ERIC-PCR was evident in this study. However, the question has been raised as to whether the ERIC-PCR products are from true ERIC sequences (Gillings and Holley, 1997). The points raised were that **1)** multiple DNA products have been amplified by ERIC-PCR with unrelated template DNA e.g. lambda bacteriophage; **2)** less than 50 copies of the ERIC sequences have been identified in *E. coli* genome. On average, therefore, there should be an ERIC sequence in every 100 kb as the total genome is 4.8×10^3 kb. However, the amplified products are less than 3 kb in size. In spite of these observations and uncertainties this method still has the merit of applicability to many bacteria as a typing method due to its reproducibility (Gillings and Holley, 1997). REP- PCR produced the highest discrimination between the *H. ovis* isolates in contrast to the similar organism *H. somnus* that showed the highest discrimination with ERIC-PCR (**Table 3.11**). The variation in patterns of amplimers generated by PCR-ribotyping of *H. somnus*, *H. ovis* and *A. seminis* indicates the diversity in number and structure of ribosomal operons in these species. Such polymorphisms have been reported from other bacterial species (Gurtler and Stanisich, 1996). The results indicated that *H. somnus*, *H. ovis* and *A. seminis* could be identified by any one of these PCR methods, but if the results of each PCR method were combined, *H. somnus* and *H. ovis* isolates would be clearly differentiated and subtyped. With all three typing methods, these three species produced different banding patterns, which clearly differentiated them from other species of the family *Pasteurellaceae* such as *P. haemolytica*, *P. trehalosi* and *P. multocida* as well as *A. pleuropneumoniae* and other unidentified isolates from bovine and ovine reproductive tracts.

Identical patterns were given by all three fingerprinting methods for the *H. somnus* isolates from three bulls of the same herd indicating the dissemination of a single strain within the herd. Similar observations were obtained with different colonies from the same swab which gave identical REP-PCR patterns and these were identical to those of isolates from different sites in the same animal (see **Figure 3.9**). The same phenomenon was observed with *H. ovis* and *A. seminis* isolates which showed persistence of a single strain in the same animal for a long time, or reinfection with the same strain. These observations provide good evidence for the reproducibility of the typing methods.

Histophilus ovis is an ovine pathogen that can cause epididymitis and orchitis. It may well be present in the same clinical specimens as *A. seminis* and it may be difficult to distinguish biochemically between the two species. As discussed earlier, Stephens *et al.*

(1983) distinguished two isolates of *A. seminis* from the *Haemophilus-Histophilus* group by their lack of yellow pigment, production of catalase and differences in cell wall envelope protein profiles. Recent genetic studies have shown a clearer picture of species differentiation, for example by *Bam*H1 restriction endonuclease profiles (McGillivery *et al.*, 1986) and DNA-DNA hybridisation techniques (Walker *et al.*, 1985; Piechulla *et al.*, 1986). In 1990, Sneath and Stevens (1990) defined the properties of *A. seminis* and proposed it as a new species on the basis of cultural, biochemical and DNA-DNA hybridisation methods. These results show that PCR methods can readily be used to differentiate between *A. seminis* and the *Haemophilus-Histophilus* group.

The first reported isolate of *H. ovis* (SA24) in the UK, used as reference strain for *H. ovis* in this study, appears to be unique and therefore perhaps not the best choice of reference. Type strains of *H. somnus* and *A. seminis* however, despite different geographic origins from the other strains in this study, generally fell into the largest group of Scottish strains. Among the *H. somnus* isolates examined, 14 were lung isolates of which seven had an identical pattern by each typing method (**Table 3.6**). This pattern was identical to that of the type strain of *H. somnus* which is of North American origin (Wisconsin State). The lung isolates studied here were from different regions of Scotland. According to D. J. Taylor (personal communication), both animals and semen have been brought to Scotland from Wisconsin State to upgrade the Scottish cattle herd and so it is possible that at least some of the *H. somnus* isolates originated from North America. Variations in PCR profiles were much higher between genital isolates than between respiratory isolates. More importantly, genital and respiratory isolates were clearly separated by all three PCR typing methods, which suggests that distinct strains inhabit these different sites. Isolates SA21, SA22 and SA23 were genital isolates from three different bulls of the same herd and they could not be differentiated by the three typing methods. This finding suggests dissemination of a single strain within the herd.

The close relationship among *H. ovis* isolates revealed by the typing methods indicates that there are only a limited number of distinct strains in Scottish sheep flocks. If respiratory and reproductive isolates had been examined, however, as for *H. somnus*, a greater diversity of types might have been demonstrated. It would be of interest to compare the diversity of fingerprint types in isolates from other geographic regions in the UK and elsewhere.

All three PCR methods produced common markers for all *A. seminis* isolates as well as producing strain-specific bands. This indicates that these PCR methods could be used for strain differentiation of *A. seminis* for epidemiological studies and also for confirmation of the identity of *A. seminis* isolates by the presence of these common markers. ERIC-PCR

was more discriminatory than REP-PCR. With *A. seminis* isolates, the types generated by the PCR methods showed no correlation with breed of sheep or disease condition of the host. However, several of these isolates from Southern Scotland obtained over a three to four year period, possessed similar fingerprints, but the number of isolates examined was not sufficient to draw clear conclusions. However one significant finding was that isolates from the same animal, taken at different times, showed reproducible fingerprints (**Table 3.10**).

Molecular methods for differentiation of *H. somnus*, *H. ovis* and *A. seminis*. McGillivery *et al.* (1986) fingerprinted the total DNA from *H. ovis* and related bacteria, including *H. somnus* and *A. seminis*, by *Bam*H1 restriction enzyme assay (REA). That study included 22 *H. ovis*, four *H. somnus* and one *H. agni* isolates from different anatomical sites and geographic regions of the world. They observed 10 common bands for the members of H-H group although the patterns were rather complex. Individual isolates of the H-H group tested were differentiated on the basis of their unique banding profiles and *A. lignieresii, A. seminis, H. somnus, H. ovis, H, agni, H. influenzae, H. parainfluenzae* and *H. parahaemolyticus* were differentiated from each other by this technique. No correlation between banding profiles and the site of isolation of *H. ovis* isolates was found. In another study, *Taq*1 REA profiles of 100 isolates of *H. somnus* were examined by Fussing and Wegener (1993). They distinguished 14 different types from 80 pneumonic isolates, of which 64 isolates were grouped into the same type, and 17 different groups from 20 genital isolates. These data indicate considerable heterogeneity within the species. This is also indicated by the data reported here. A similar observation was made by Kirkham *et al.* (1989) but they differentiated only a single *H. somnus* isolate from a single *H. ovis* isolate by restriction endonuclease profiles generated by *Hind*III, *Eco*RI-*Hind*III, *Pst*1 and *Hha*1. There were marked differences of restriction endounclease profiles between *H. somnus* and *H. ovis* isolates in a recent study (Ward *et al.*, 1995). Six *H. somnus* and 12 *H. ovis* isolates were included and, by *Hin*f1 restriction, they differentiated five types among six *H. somnus* isolates and 10 types of 15 *H. ovis* isolates. Ribotyping is the other DNA based molecular technique that has been applied for differentiation of *H. somnus* and *H. ovis* isolates. In one study, 13 ribotypes have been obtained for 100 *H. somnus* isolates with rRNA-probed *Eco*R1 restriction fragments (Fussing and Wegener, 1993). Seven different ribotypes were observed among 80 pneumonic strains. Seventy nine per cent of these showed a similar pattern and the remaining 21% showed six different types. The reproductive isolates (n=20) divided into 11 different types. This indicates that Danish reproductive isolates of *H. somnus* were more heterogeneous than respiratory isolates which is a similar situation to that of Scottish isolates. The other study for ribotyping of *H. somnus* and *H. ovis* produced four types and eight types respectively (Ward *et al.*, 1995). Among six isolates of *H. somnus*, each isolate

from cases of TEME, pneumonia and myocarditis showed the same type while three isolates of reproductive origin (one abortion and two preputial) had distinctive patterns. The three *H. ovis* isolates from cases of septicaemia were the same ribotype and the rest of the isolates showed a distinct pattern except two which had the same pattern (Ward *et al.*, 1995). In the present study, the results of PCR-ribotyping clearly differentiated all but three of the *H. ovis* isolates from *H. somnus* by the presence of a dual band of about 0.7 kb. Nevertheless, three sheep strains (*H. ovis*) had the same PCR-ribotype as one of the *H. somnus* types, suggesting that the host-specific relationship may not be absolute.

Among the 24 *A. seminis* isolates there were two ribotypes, five REP types and nine ERIC types. With this study, genetic heterogeneity among *A. seminis* was revealed, although it has been reported that *A. seminis* is genetically homogeneous by *Bam*H1 restriction endonuclease profiles (McGillivery and Webber, 1989).

Value of PCR methods for differentiation of species and strains. PCR as a means of fingerprinting of *H. somnus*, *H. ovis* and *A. seminis* isolates has only previously been applied in a RAPD study (Myers *et al.*, 1993). For this they used 16 isolates of *H. somnus* and one isolate each of *H. ovis, H. agni* and *A. seminis*. For the application of this technique, 16 different decamer random primers were tested and these produced 0-14 amplified bands for each isolate. The results of only nine primers were used for strain differentiation as the PCR products of the other primers were not useful for analysis. *H. somnus* isolates produced similarity coefficients between 0.46 and 1.00 on the basis of pair-wise comparisons of RAPD amplimers produced with nine random decamer primers. A higher correlation between encephalitic and respiratory isolates was reported with this method and a poor correlation among reproductive isolates. With this RAPD method, *H. somnus*, *H. ovis* and *A. seminis* species were clearly distinguished. As in REP-PCR, ERIC-PCR and PCR-ribotyping, the complexity of the RAPD fingerprints are dependent on the primers used. The RAPD technique is comparatively technically difficult, as RAPD needs the selection of primers which in turn is time consuming and expensive. Although the RAPD technique is discriminative, the reproducibility of this technique has been questioned (Meunier and Grimont, 1993). Power (1996) doubted the reproducibility of this RAPD technique he suggested that stringent standardisation of the method would help to achieve good reproducibility. The great advantage of the PCR fingerprinting methods is that they allow the production of rapid and discriminatory fingerprints without any knowledge of the genome of the organism. In this study, the banding profiles were relatively complex for REP and ERIC-PCR, and the significance of less intense band differences needs to be established, but PCR-ribotyping produced a simpler pattern and a smaller number of distinct types.

PCR-ribotyping produces a simple pattern in general. In fact, this PCR-ribotyping pattern should directly correlate with the number of ribosomal operons in the bacterial genome. So far, the highest number of operons found in the bacterial genome is 12 in *Haemophilus influenzae* and 10 in *Bacillus subtilis* (Gurtler and Stanisich, 1996). Theoretically the number of bands would be similar to the number of ribosomal operons in the bacterial genome but the band number may be increased as a result of non-specific PCR amplification of other regions. Significantly, the PCR-ribotyping pattern for *A. seminis* was similar in all the strains, except SA33 strain which produced a clear additional band. The PCR-ribotyping patterns for *H. ovis* and *H. somnus* were entirely different from those of *A. seminis* which indicates that PCR-ribotyping can be used to identify *A. seminis* and to differentiate it from these other species. The PCR-ribotyping results also indicated that there are at least two ribosomal operons in the genome of *A. seminis*. In contrast, the same PCR typing method with *H. somnus* and *H. ovis* produced eight and five types respectively indicating a greater number of ribosomal operons in these species.

In some reports, the similar PCR-ribotyping amplimers from different isolates have been digested with restriction enzymes in order to differentiate these similar patterns. One organism that gave similar but simple PCR-ribotyping fingerprints was *Burkholdaria cepacia* (Ryley *et al.*, 1995). The amplimers of PCR-ribotyping were extracted and digested with *Taq*I, *Rsa*I and *Hpa*II. The *Taq*I-digested PCR products yielded seven different electrophoretic patterns by which epidemic strains of *Burkholdaria cepacia* were easily differentiated from reference strains. A similar method has been applied to differentiate *Chlamydia* species (Meijer *et al.*, 1997). The PCR-ribotyping of different *Chlamydia* species produced a single 0.803 kb PCR product. Digestion of this PCR product with *Mse*I restriction enzyme differentiated all four *Chlamydia* species and it also subdivided *Chlamydia* trachomatis into a human and a murine group, and *C. psittaci* was subdivided into an avian and feline group. If the PCR-ribotyping amplimers of *A. seminis* had been digested with suitable restriction enzymes, the discrimination between strains could probably have been increased.

REP- and ERIC-PCR produced complex patterns for the three species. The analysis of fingerprints was done visually. This procedure is simple but subjective and prone to errors. Alternatively, computer assisted gel documentation methods have been developed for analysis of such fingerprints. In a RAPD analysis of *Acinetobacter baumannii*, fluorescently labelled primers were used to amplify PCR products. These labelled PCR products were analysed by a high-speed automated fluorescent DNA sequencing machine which generate the digitalised data which can be seen as a densitogram on the computer screen. From these on line data, a dendrogram may be prepared. The other advantages of this method are that analysis of large number of strains (n=40) with complex fingerprints

containing small fragments can be done more accurately and at high-speed (5-8 h) than with conventional agarose gel electrophoresis (Grundmann *et al.*, 1995). With this method, the phylogenetic analysis of isolates is very much easier than by the pair-wise comparison method adopted by Myers *et al.* (1993) for the analysis of similarity co-efficients of these three species by RAPD. Snelling *et al.* (1996) used different methods to analyse fingerprints in addition to visual inspection. Computer software called Dendron software (Soiltech, Inc., Oakdale, Califonia) was used to analyse the scanned gel photographs.

Molecular techniques are very useful tools for bacterial typing. Restriction endonuclease analysis of chromosomal DNA using agarose gel electrophoresis often generates complex fingerprints which are difficult to interpret. Smaller fragments show poor resolution and cannot be used to discriminate between strains (Fussing and Wegener, 1993). Conventional ribotyping requires restriction endonuclease digestion of the bacterial DNA, Southern blotting, hybridisation with ribosomal DNA-directed probes and subsequent autoradiography. This procedure needs several days and the reported typability of *H. somnus* strains was 95% (Fussing and Wegener, 1993). The arbitrary primed PCR (AP-PCR, RAPD) uses short randomly chosen oligonucleotide primers to create species- and strain-specific DNA fingerprints, but may require the initial testing of many individual primers. REP-PCR, ERIC-PCR and PCR-ribotyping have been used to identify many bacterial species and also to differentiate strains within species of both Gram-positive and Gram-negative bacteria (Versalovic *et al.*, 1991; Jensen *et al.*, 1993). These methods show several valuable characteristics: there is no necessity for strain, species or genus specific probes; they allow the use of a single set of primers in each method for the analysis of closely related as well as widely divergent strains; no Southern blotting or DNA hybridisation experiments are required to analyse the PCR products and simple agarose gel electrophoresis is sufficient; and boiled cell extracts as template DNA produced the same results as extracted chromosomal DNA for all three PCR methods (Kerr, 1994).

Use of simple cellular extracts as template DNA. PCR-based DNA fingerprinting methods originally used extracted chromosomal DNA as template DNA (Versalovic *et al.*, 1991; Jensen *et al.*, 1993). These extraction methods involve the use of phenol-chloroform. The use of these chemicals is not only a safety hazard but also remnants of these chemicals may inhibit the PCR reaction. Alternatively, there are commercial kits available for extraction of chromosomal DNA without using those chemicals but these kits are expensive. As a substitution, boiled whole cell extracts have been successfully used as template DNA (Kerr, 1994). The use of boiled cell extracts was validated for REP-PCR for the typing of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex (Snelling *et al.*, 1996). This method has now been successfully applied to the fingerprinting of *H. somnus* by PCR methods including REP-PCR (Appuhamy *et*

al., 1997). It was shown that boiled cell extracts as template DNA produced the same results as extracted chromosomal DNA for all three PCR methods. In the same way it has been shown in this study to work well for *H. ovis* and *A. seminis* species.

PCR-based fingerprinting is simple and rapid and can be performed with very small quantities of bacterial cultures. It also allows 100% typability of isolates. Reproducibility was excellent with all three methods although some day-to-day variation in the intensity of amplified fragments, particularly the minor bands, was observed. As has been reported earlier (Chatelut *et al.*, 1995), annealing temperature, extension time, magnesium ion concentration and the number of cycles had an effect on the banding profiles in terms of intensity and number of the bands and this must be strictly controlled. Even so, some day to day variation was seen in band intensity, presumably due to slight changes in reaction conditions and perhaps to slight differences between batches of reagents. For a rigorous comparison of fingerprints of different strains, the PCR should be done at the same time, with same reagents and amplimers run side by side on same gel. For gel to gel comparisons, it is useful to have the same "control" sample of known profile run on the different gels.

One source of concern was the variation of REP-PCR fingerprints with the change of primer batch. This may be due to the presence of 'N' (any of A/T/C/G; A, adenine, T, thymine, C, cytosine, G, guanine) and 'I', inosine (**Table 2.4**) in these primer sequences. A similar argument was raised by Lipman *et al.* (1995) where they explained the poor reproducibility due to the presence of deoxyinosines in the REP primers. At 'N' any of the four bases may be incorporated giving a complex mixture of sequences in the primer and a mixture of different composition in each batch. This may lead to a change of hybridisation to the template DNA resulting in different amplifications and different fingerprints. The same applies to 'I' as it can bind to any of the four bases, leading to mismatching. For the sake of reproducibility, it would probably be best to specify the sequences of the REP primers omitting 'N's and 'I's. This might have the effect of reducing the number of amplimers and thereby simplifying the band patterns.

4.5 PLASMID PROFILES

The plasmid profiles of the isolates showed that a high proportion of *H. ovis* isolates (63%) had plasmids and the profiles of most of the isolates were similar. Their sizes ranged from 3.0 to 5.0 kb. A relatively smaller percentage of *H. somnus* (13%) and *A. seminis* (8%) isolates had plasmids. All plasmids of *H. somnus* isolates were single plasmids, with sizes from 1.5 to 3.5 kb. Both *A. seminis* strains with plasmids showed two plasmids but

their sizes were different, from 2.6 to 4.9 kb. However, the plasmid profile of one *A. seminis* strain was similar to that of most of the *H. ovis* isolates. This similarity may be a result of plasmid transfer between *H. ovis* and *A. seminis* as these two species are common inhabitants of the ram reproductive tract. In another study, all *H. ovis* isolates tested had plasmid profiles but none of the *H. somnus* isolates contained plasmids (Kirkham *et al.*, 1989) and they used this method to differentiate *H. somnus* from *H. ovis* but this study would indicate that this is not satisfactory as not all the *H. ovis* strains contained plasmid DNA and some *H. somnus* isolates did. The *H. ovis* isolates were reported to contain from one to six plasmids ranging in size from 3.9-90 kb. In contrast to this finding, Fussing and Wegener (1993) found 20% of Danish *H. somnus* isolates contained plasmids. The predominant sizes of the plasmids were 1.5-2.5 kb. Fifteen per cent of pneumonic isolates showed plasmids with seven different profiles and 40% of the reproductive isolates had plasmids with six profiles. There are no reports of plasmid isolation from *A. seminis* isolates. Except for the findings of Kirkham *et al.* (1989) plasmid profile analysis as a molecular fingerprinting method appears to have limited value since only a small proportion of strains contain plasmids.

4.6 ANTIBIOTIC SENSITIVITY PATTERNS

The antibiotic sensitivity patterns of the isolates showed that they were sensitive to most of commonly used antibiotics. According to the sensitivity patterns, *A. seminis* isolates were differentiated from the other two species as all *A. seminis* isolates tested were sensitive to cotrimoxazole whereas the majority of *H. somnus* and *H. ovis* isolates were resistant. In general, the profiles within each species were the same. There was no evidence of multiresistance. However, the number of antibiotics tested was limited and not all isolates were assessed for their antibiotic sensitivity. These findings showed that the plasmids found in some strains have no apparent relation to any antibiotic resistance. A similar finding was made earlier by Sugimoto *et al.* (1983) who reported resistance of *H. somnus* to some antibiotics but they excluded the involvement of plasmids for the resistance.

4.7 RIBOSOMAL OPERONS OF *A. SEMINIS*

The sequencing of the two PCR-ribotyping products uncovered some useful information about the *A. seminis* genome. The results indicated that there are at least two ribosomal operons. The *rrn*A sequence contains a gene for tRNA-Glu in the spacer region and the *rrn*B sequence contains genes for tRNA-Ile and tRNA-Ala in the spacer region.

Gurtler and Stanisich (1996) studied the bacterial ribosomal operon spacer regions in great detail. They found that sequence homology was apparent only in the regions where the tRNAs were encoded. However, these tRNA genes are not present in the 16S and 23S spacer regions of all species. As the sequence homology is poor in the spacer region outside the tRNA genes, they suggested that this region would be a good target for design of species-specific primers for specific identification of bacteria by PCR. The sequences of the two spacer regions of these two operons were aligned with their counterparts in some other bacterial sequences that were analysed by Gurtler and Stanisich (1996) (**Appendix 5** and **6**). Analysis of the 16S and 23S spacer region sequences showed that tRNA-Glu was normally found alone and not with either tRNA-Ile or tRNA-Ala. In other bacteria, tRNA-Ile and tRNA-Ala may be present together in a single spacer region or they may occur separately. Some spacer regions do not contain any of these tRNAs.

The sequence similarities of the two RNA operons of *A. seminis* were restricted to the 16S and 23S RNA and tRNA genes. The highest similarity was observed with those of *Haemophilus influenzae* where the *A. seminis* 16S rRNA, tRNA-Glu and tRNA-Ile sequences were identical and the 23S rRNA and tRNA-Ala sequences showed 97% similarity with those of *H. influenzae*. The sequences of these tRNA genes from different bacterial species were compared with those of *A. seminis* in **Appendix 7** which shows that the variation of sequences within the tRNA gene are limited to particular points of the tRNA.

4.8 IDENTIFICATION OF *A. SEMINIS* BY PCR

Use of PCR for detection of bacterial pathogens. The detection of human and animal pathogens by PCR has been achieved with specimens of many biological fluids, including cerebrospinal fluid, blood, sputum, tears, middle ear fluid, chancroid swabs, urine and semen (Whelen and Persing, 1996). With few exceptions, PCR samples have been prepared from these specimens by organic extraction and ethanol precipitation of the DNA, a labour intensive process involving multiple steps. Therefore, simplified sample preparation methods, such as those described in this study, are particularly useful for the rapid PCR-based diagnosis of infected samples. The design of primers has varied. Most studies have targetted either species-specific genes, such as *tox*A of *P. multocida* (Nagai *et al.*, 1994; Lichtensteiger *et al.*, 1996) or the urease subunit gene of *Helicobacter pylori* (Furuta *et al.*, 1996), or have used species-specific probes derived from rRNA sequences (Romero *et al.*, 1995). Fewer studies have used primers designed from the spacer region between the 16S and 23S genes of the ribosomal operon (Smart *et al.*, 1996; Tilsala-

Timisjarvi and Alatossava, 1997) although the ribosomal intergenic regions are more variable between species than the rRNA genes themselves (Barry *et al.*, 1991).

The path for the development of *A. seminis*-specific PCR. The PCR-ribotyping of *A. seminis* in this study had shown that amplification of the spacer region between the 16S and 23S rRNA genes of 24 *A. seminis* isolates revealed a characteristic pattern of two high intensity amplimers of 0.55 and 0.7 kb. This PCR-ribotype was distinct from that of other species isolated from the ovine and bovine reproductive tracts such as *H. somnus* and *H. ovis*. Primers SRJAS1 and SRJAS2, designed from the spacer region of *rrn*B, were shown to be specific for *A. seminis* and no amplification products were obtained from any other Gram-negative species tested including *H. somnus*, *H. ovis* and several other strains of unknown identity isolated from the bovine and ovine reproductive tracts.

Reduction of inhibition of PCR. A major barrier to the direct use of body fluids in PCR is the presence of inhibitory components in the samples. Two procedures were adopted here to reduce the inhibition of the PCR process. The incorporation of the chelating resin Chelex® 100 into the sample preparation process has been shown to increase the sensitivity of PCR (Walsh *et al.*, 1991; de Lamballerie *et al.*, 1992) and this was confirmed in the the present study. In addition, presumptive inhibitory protein components of the storage solution were reduced by treatment with proteinase K (Domeika *et al.*, 1994). PCR of semen samples artificially infected with *A. seminis* showed that it was only necessary to dilute the semen 10-fold in order to obtain a clear positive result and in a similar study, a 10-fold dilution of semen had been used for detection of *Chlamydia psittaci* by PCR (Domeika *et al.*, 1994). However, the presence of semen storage solution decreased the sensitivity of the PCR reaction and it was necessary to treat samples with proteinase K and boil them in the presence of Chelex 100 in order to achieve a sensitive assay. Thus, fresh semen without added storage solution would be a better substrate for *A. seminis* diagnosis by PCR because of the inhibitory effect of the storage solution and the 1:3 dilution of semen with storage solution reduces the available template for PCR in the sample. These experiments indicated that the inhibitory effect was due to the storage solution and not the seminal plasma, which contrasts with the findings of Guerin *et al.*(1995) who noted an inhibitory effect due to the seminal plasma of boar semen. Under optimal conditions, the method allowed the detection of approximately three CFU per 10 µl sample (300 CFU/ml) containing 100 fold diluted semen without storage solution.

***A. seminis*-specific PCR assay.** The PCR assay with primers SRJAS1 and SRJAS2 may be used for detection and identification of *A. seminis*. The advantages of this PCR assay are its rapidity and specificity. As *A. seminis* is a fastidious and slow-growing

bacterium, the primary isolation and presumptive identification takes several days. With this PCR method, it can be identified within 12 h of primary isolation. The differentiation of *A. seminis* from phenotypically similar organisms such as *Histophilus ovis,* which may be present in the same clinical specimens and which are also associated with epididymitis, is sometimes difficult, and there have been cases of misidentification (Walker *et al.*, 1986). So far, there is no definitive biochemical test for the specific identification of *A. seminis* and the use of specific primers in the PCR assay should provide a rapid diagnostic test without the need to culture the organism. The sensitivity of the assay is such that semen samples contaminated with low levels of *A. seminis* may not give a positive result, but it should be sensitive enough to detect *A. seminis* in moderately contaminated semen specimens where the organism is causing clinical problems. On the other hand, this molecular method for identification would not eliminate the need for culture of *A. seminis.* Obviously, it has to be cultured and propagated in order to store it for future reference and, at present, live bacteria are still necessary for antibiotic sensitivity assays.

4.9 CONCLUSIONS AND PERSPECTIVES

There is an increasing awareness of the importance of *H. somnus*, *H. ovis* and *A. seminis* as pathogens of cattle and sheep and reports of isolation of these organisms are increasing. Nevertheless, relatively little work has been done to characterise these species, to elucidate their virulence factors and pathogenic mechanisms and to determine their taxonomic position in the microbial world.

H. somnus, *H. ovis* and *A. seminis* are phenotypically similar organisms and their identification by conventional means is often uncertain and time consuming. Indeed, these organisms are fragile and fastidious in their growth requirements and isolation may not always be successful, particularly if there is any delay in transport of specimens to the laboratory. For this reason, identification procedures such as those involving PCR, which do not rely on culture, would be beneficial. Even when the organisms have been identified, there are no generally accepted methods for typing of these strains for epidemiological studies, for example by biochemical, serological or phage-typing methods. In addition, there is some confusion surrounding the identity and nomenclature of *H. somnus* and *H. ovis*. They appear to be closely related and cause similar disease syndromes in cattle and sheep respectively. These include reproductive disorders such as orchiepididymitis, endometritis and abortion and other diseases including thromboembolic meningoencephalitis, pneumonia, septicaemia and arthritis. *A. seminis* causes reproductive problems in rams and may be present in the same clinical specimen as *H. ovis*.

The present study was designed to improve the methods available for identification and typing of these species and the focus of interest was to apply molecular methods, namely PCR fingerprinting, to these problems. Molecular methods that are based on the analysis of the microbial genome have the advantage that they are free from most of the difficulties associated with phenotypic methods which tend to be time consuming, labour intensive, inconsistent and with poor discrimination. DNA based methods offer a more refined and reproducible means to differentiate strains. PCR-based methods are especially attractive because of their speed and simplicity. The other great advantage of PCR over other identification methods is that the viability of the test organism is not necessary and the quality of specimens should not be a problem for diagnostic purposes.

Previous findings (Stephens *et al.*, 1983) concluded that *H. somnus* and *H. ovis* are similar organisms which should be grouped in to a Haemophilus-Histophilus group, along with another similar bacterium *Haemophilus agni*. It was also concluded that *A. seminis* was distinct biochemically, antigenically and cytochemically from the members of H-H group. The present study confirmed the findings of Stephens *et al.* (1983). *A. seminis* was clearly differentiated from the other two species by all three PCR methods and the fingerprinting results showed some relationship between *H. somnus* and *H. ovis*. By REP- and ERIC-PCR, *H. somnus* and *H. ovis* were similar but distinct species and PCR-ribotyping clearly differentiated these two species, except for three strains of *H. ovis* which were similar to *H. somnus* although of ovine origin. In further studies, it would be of interest to isolate more of these *H. somnus*-like ovine strains and to address their relationship to typical bovine *H. somnus* and ovine *H. ovis*, perhaps by analysis of 16S rRNA sequences. This type of analysis has successfully been applied to analyse different strains of *Yersinia* from raw milk samples (Ibrahim *et al.*, 1997). With such data, dendrograms could be produced to analyse the evolutionary relationship between *H. somnus* and *H. ovis*. It would also be of interest to determine the virulence of these three strains along with typical bovine *H. somnus* and ovine *H. ovis* in sheep and cattle by various routes e.g. respiratory and reproductive although, as yet, there is no well characterised model for testing virulence of these species.

Keeping to the objectives of this study, the developed PCR techniques are readily applicable to the identification of *H. somnus*/*H. ovis* and *A. seminis*. These methods should prove to be highly useful for the identification and differentiation of *H. ovis* and *A. seminis* from the same clinical specimens i.e. ram semen. The rapid fingerprinting methods developed in this study should also be useful for epidemiological studies of *H. somnus*, *H. ovis* and *A. seminis*, to follow the spread of particular strains in the cattle and sheep populations. The other use of these methods will be to determine whether a particular type is more closely associated with diseased or healthy animals. In the present study, with a

relatively limited number of isolates, no such evidence was found and no attempt was made to relate 'types' to virulence properties possessed by the strains and whether a 'virulence' phenotype correlated with a particular genetic fingerprint. Nevertheless, if pathogenic types could be identified, this would help in the identification and characterisation of virulence factors in these organisms. If no such clear relationship exists, this would suggest that host factors are more important than bacterial factors in determining whether or not the disease occurs.

Another molecular typing method investigated in present study was plasmid profile analysis. This method was found to have little value for *H. somnus* and *A. seminis* since comparatively few isolates had plasmids but it was more useful with ovine *H. ovis* where the majority of strains contained one to two small plasmids of different sizes. Investigation of the relationship of plasmids to antibiotic resistance of these isolates could be considered as inconclusive since only a limited number of antibiotics were tested.

In sampling of bovine reproductive tracts for *H. somnus*, an isolate was obtained which, by cultural, biochemical and PCR-fingerprinting was indistinguishable from *A. seminis* strains from rams. This finding deserves further investigation and could have implications for transmission and pathogenesis of disease caused by *A. seminis*. A large number of bovine reproductive tracts should be screened to determine the prevalence of this organism and its association with pathological conditions. The isolation of *A. seminis* from bull semen has been reported (Dixon *et al.*, 1983) but it is not clear whether that isolate was a true *A. seminis*. Again studies on virulence of such bovine isolates in rams would be of great interest and relevant to the possible transmission of *A. seminis* between sheep and cattle.

A definitive identification of *A. seminis* with available techniques is difficult. *A. seminis* causes severe permanent damage to the reproductive organs of rams, with a heavy economic loss to sheep farms. Rapid diagnosis of *A. seminis* would be useful for early treatment and to reduce the damage caused by the infection. Early isolation of infected animals would also prevent the spread of the disease. The *A. seminis*-specific PCR based diagnostic test developed in the present study would be very useful for rapid and specific identification of *A. seminis* in suspected semen samples and could be directly applied to routine screening of samples before storage or distribution of semen for artificial insemination.

The DNA sequences data of the 16S-23S spacer regions of *A. seminis*, deposited in the Genbank, will be beneficial for comparative studies with similar regions of other bacteria. These sequences revealed that the *A. seminis* genome contains at least two rRNA

operons, one of which contains a single tRNA (for glutamine) and the other contains two tRNAs (for isoleucine and alanine).

Bearing in mind the fastidious nature of the bacteria studied in this investigation, an important objective was to develop a PCR-based mean of direct identification of the organisms without primary culture. These objectives were met for *A. seminis* as described above and the current work has laid the foundation for a similar approach with *H. somnus* and *H. ovis*. For example, from the PCR-ribotyping profiles of these organisms, common and unique bands are visible. Thus, it would be possible to sequence and design primers from these bands which could be used for PCR-based identification of *H. somnus* and *H. ovis* together and also to differentiate between them.

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6. APPENDICES

Appendix 1 ***API ZYM tests and the corresponding substrates***

Test	Enzyme assayed for	Substrate
1	Control	-
2	Phosphatase alkaline	2-naphthyl phosphate
3	Esterase (C 4)	2-naphthyl butyrate
4	Esterase Lipase (C 8)	2-naphthyl caprylate
5	Lipase (C 14)	2-naphthyl myristate
6	Leucine arylamidase	L-leucyl-2-naphthalamide
7	Valine arylamidase	L-valyl-2-naphthalamide
8	Cystine arylamidase	L-cystyl-2-naphthalamide
9	Trypsin	N-benzyl-DL-arginine-2-naphthalamide
10	Chymotrypsin	N-glutaryl-phenylalanine-2-naphthalamide
11	Phosphatase acid	2-naphthyl phosphate
12	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate
13	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside
14	β-galactosidase	2-naphthyl-βD-galactopyranoside
15	β-glucuronidase	Naphthol-AS-BI-βD-glucuronide
16	α-glucosidase	2-naphthyl-αD-glucopyranoside
17	β-glucosidase	6-Br-2-naphthyl-βD-glucopyranoside
18	N-acetyl-β glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide
19	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside
20	α-fucosidase	2-naphthyl-αL-fucopyranoside

Activity of the enzymes are scored accoding to the quantity of the hydrolysed substrate in nanomoles.

0 for no activity or 0 nanomoles.

1 as 5 nanomoles.

2 as 10 nanomoles.

3 as 20 nanomoles.

4 as 30 nanomoles.

5 as ≥40 nanomoles.

Appendix 2 *Enzyme activity of H. somnus isolates in API ZYM system*

Isolate	Activity of enzyme on API ZYM substrates (Test No.)																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
THs	0	1	1	1	0	4	0	0	0	0	3	1	0	0	5	0	0	0	0	0
SA01	0	5	2	2	0	4	0	0	0	0	5	4	0	0	5	0	0	0	0	0
SA02	0	3	1	1	0	5	0	0	0	0	5	3	0	0	5	0	0	0	0	0
SA03	0	4	2	3	0	4	0	0	0	0	5	3	0	0	5	0	0	0	0	0
SA04	0	4	3	3	0	5	1	0	0	0	5	3	0	0	5	0	0	0	0	0
SA05	0	4	3	3	0	5	1	0	0	0	5	3	0	0	5	0	0	0	0	0
SA06	0	3	1	2	0	5	1	0	0	0	3	3	0	0	5	0	0	0	0	5
SA07	0	2	2	2	0	5	1	0	0	0	4	1	0	0	5	0	0	0	0	0
SA11	0	3	3	3	0	5	0	0	0	0	5	4	0	0	5	0	0	0	0	0
SA12	0	3	1	2	0	4	0	0	0	0	2	3	0	0	5	0	0	0	0	1
SA13	0	5	1	2	0	5	1	0	0	0	5	3	0	0	5	0	0	0	0	0
SA14	0	3	3	3	0	5	1	0	0	0	4	3	0	0	5	0	0	0	0	5
SA15	0	3	3	3	0	5	0	0	0	0	5	4	0	0	5	0	0	0	0	5
SA17	0	2	2	2	0	5	0	0	0	0	4	1	0	0	5	0	0	0	0	1
SA19	0	2	2	2	0	4	0	0	0	0	4	1	0	0	5	0	0	0	0	0
SA20	0	3	3	3	0	4	0	0	0	0	5	1	0	0	5	0	0	0	0	0
SA21	0	1	1	1	0	4	0	0	0	0	3	1	0	0	5	0	0	0	0	0
SA22	0	1	1	1	0	4	0	0	0	0	2	1	0	0	5	0	0	0	0	0
SA23	0	1	1	1	0	4	0	0	0	0	3	1	0	0	5	0	0	0	0	0
SA48	0	1	1	1	0	4	0	0	0	0	3	1	0	0	5	0	0	0	0	5
SA49	0	3	1	1	0	5	0	0	0	0	3	1	0	0	5	0	0	0	0	0
SA50	0	1	1	1	0	4	0	0	0	0	4	1	0	0	5	0	0	0	0	1
SA51	0	2	0	1	0	4	1	0	0	0	3	1	0	0	5	0	0	0	0	0
SA52	0	2	0	1	0	5	1	0	0	0	3	1	0	0	5	0	0	0	0	0
SA68	0	2	1	2	0	5	1	0	0	0	4	1	0	0	5	0	0	0	0	1
V3	0	4	1	1	0	5	0	0	0	0	3	1	0	0	5	0	0	0	0	0
V8	0	2	2	2	0	5	0	0	0	0	2	1	0	0	5	0	0	0	0	0
X1	0	1	1	1	0	4	0	0	0	0	3	2	0	0	5	0	0	0	0	4
X4	0	2	2	2	0	5	0	0	0	0	4	1	0	0	5	0	0	0	0	1
Ph	0	5	1	0	0	4	2	0	0	0	4	2	0	2	0	0	0	0	0	1
α-chy	0	0	5	5	0	5	0	0	4	5	0	1	0	0	0	0	0	0	0	0

Controls: Ph, *Pasteurella haemolytica*; α-chy, α-chymotrypsin.

Appendix 3 ***Enzyme activity of H. ovis isolates in API ZYM system***

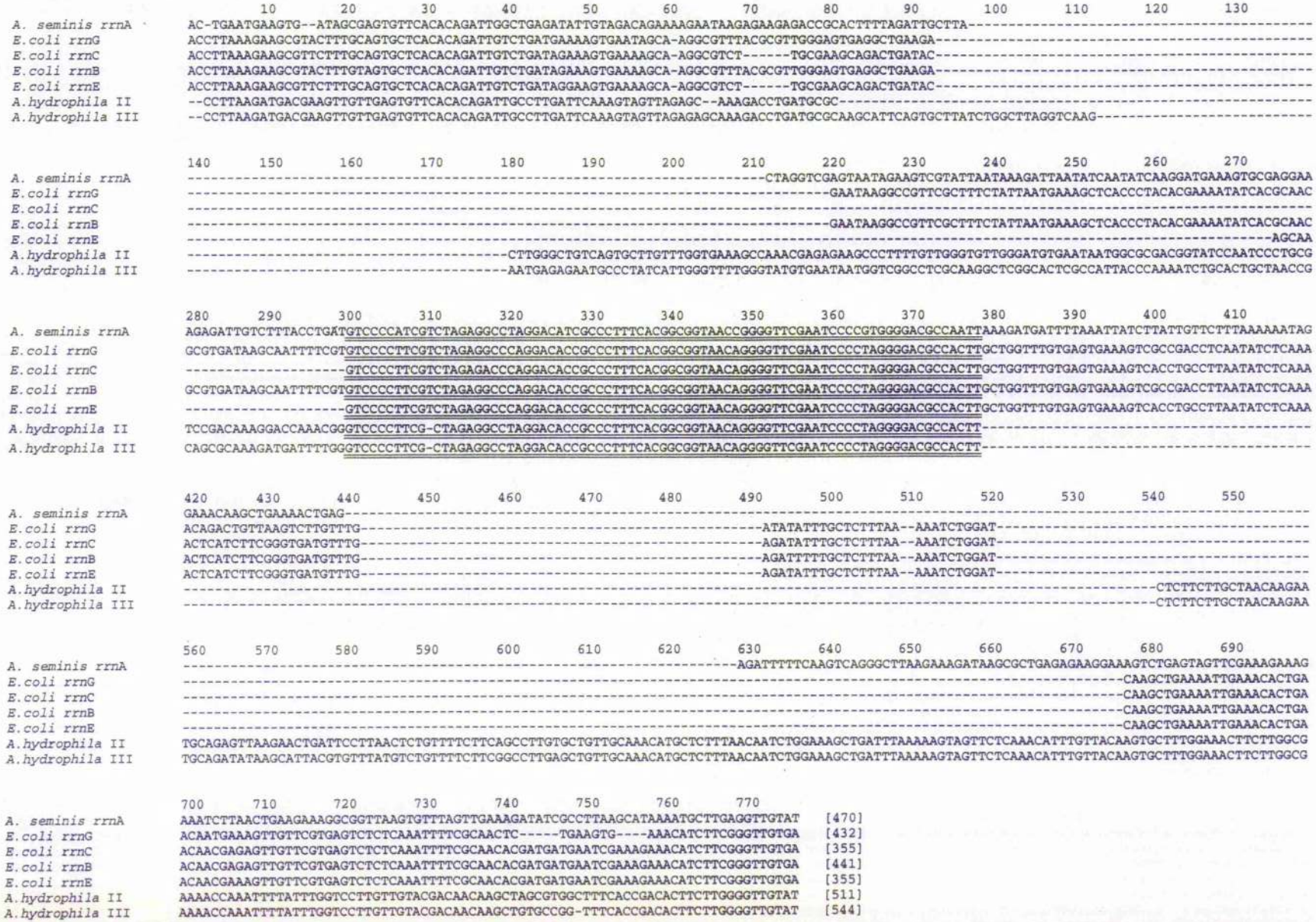
Isolate	Activity of enzyme on API ZYM substrates (Test No.)																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
SA08	0	3	2	2	0	4	0	0	0	0	5	2	0	0	5	0	0	0	0	1
SA16	0	3	2	3	0	4	0	0	0	0	5	4	0	0	5	0	0	0	0	0
SA24	0	1	0	1	0	3	0	0	0	0	2	1	0	0	5	0	0	0	0	1
SA26	0	1	0	1	0	2	0	0	0	0	3	1	0	0	5	0	0	0	0	1
SA27	0	1	0	1	0	3	0	0	0	0	4	1	0	0	5	0	0	0	0	0
SA28	0	3	0	1	0	3	1	0	0	0	4	1	0	0	5	0	0	0	0	1
SA29	0	1	0	1	0	3	0	0	0	0	4	1	0	0	5	0	0	0	0	0
SA44	0	1	1	1	0	3	0	0	0	0	4	1	0	0	5	0	0	0	0	1
SA45	0	1	1	1	0	2	0	0	0	0	2	1	0	0	5	0	0	0	0	0
SA46	0	1	1	1	0	2	0	0	0	0	4	1	0	0	5	0	0	0	0	0
SA53	0	1	0	1	0	4	0	0	0	0	2	1	0	0	5	0	0	0	0	1
SA54	0	2	1	1	0	5	1	0	0	0	4	1	0	0	5	0	0	0	0	1
SA55	0	1	1	1	0	5	1	0	0	0	4	1	0	0	5	0	0	0	0	0
SA56	0	2	1	2	0	5	1	0	0	0	4	1	0	0	4	0	0	0	0	0
SA57	0	1	0	1	0	4	1	0	0	0	4	1	0	0	4	0	0	0	0	1
SA58	0	1	0	1	0	3	0	0	0	0	4	1	0	0	4	0	0	0	0	0
SA69	0	1	0	1	0	3	1	0	0	0	3	1	0	0	5	0	0	0	0	0
SA72	0	2	0	1	0	4	0	0	0	0	4	1	0	0	5	0	0	0	0	0
SA73	0	2	0	1	0	5	0	0	0	0	4	2	0	0	5	0	0	0	0	0

Appendix 4 ***Enzyme activity of A. seminis isolates in API ZYM system***

Isolate	Activity of enzyme on API ZYM substrates (Test No.)																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
TAs	0	2	1	1	0	5	0	0	0	0	4	1	0	0	5	0	0	0	0	0
SA25	0	3	0	1	0	4	0	0	0	0	5	1	0	0	5	0	0	0	0	0
SA30	0	4	0	0	0	4	0	0	0	0	5	1	0	0	5	0	0	0	0	0
SA31	0	3	0	1	0	3	0	0	0	0	4	1	0	0	5	0	0	0	0	0
SA32	0	3	0	1	0	4	0	0	0	0	4	1	0	0	5	0	0	0	0	0
SA33	0	3	0	1	0	5	1	0	0	0	4	1	0	0	5	0	0	0	0	0
SA34	0	5	0	1	0	4	0	0	0	0	5	1	0	0	5	0	0	0	0	0
SA35	0	4	1	2	0	5	0	0	0	0	5	1	0	0	5	0	0	0	0	0
SA36	0	2	0	1	0	4	0	0	0	0	3	1	0	0	5	0	0	0	0	0
SA37	0	2	0	1	0	4	1	0	0	0	4	1	0	0	5	0	0	0	0	0
SA38	0	5	0	1	0	4	1	0	0	0	5	1	0	0	5	0	0	0	0	0
SA39	0	2	0	1	0	4	0	0	0	0	4	1	0	0	5	0	0	0	0	0
SA43	0	3	1	1	0	5	0	0	0	0	5	1	0	0	5	0	0	0	0	0
SA60	0	4	0	0	0	4	1	0	0	0	4	1	0	0	5	0	0	0	0	0
SA61	0	5	0	0	0	4	1	0	0	0	5	1	0	0	5	0	0	0	0	0
SA62	0	2	0	0	0	4	1	0	0	0	5	1	0	0	5	0	0	0	0	0
SA63	0	4	0	0	0	3	1	0	0	0	5	1	0	0	5	0	0	0	0	0
SA64	0	5	0	0	0	3	1	0	0	0	5	1	0	0	5	0	0	0	0	0
SA65	0	5	0	0	0	5	1	0	0	0	5	1	0	0	5	0	0	0	0	0
SA66	0	5	0	0	0	5	1	0	0	0	5	1	0	0	5	0	0	0	0	0
SA67	0	2	0	0	0	5	1	0	0	0	5	1	0	0	5	0	0	0	0	0
SA70	0	2	0	1	0	4	1	0	0	0	5	1	0	0	4	0	0	0	0	0
SA71	0	2	0	0	0	4	1	0	0	0	5	1	0	0	5	0	0	0	0	0
X16	0	1	1	1	0	5	0	0	0	0	5	1	0	0	5	0	0	0	0	0

Appendix 5 ***Comparison of rrnA of A. seminis with the ribosomal operon of some other bacteria (Gurtler and Stanisich, 1996).*** The sequences are from the end of the 16S gene and the beginning of the 23S gene. Gaps (-) have been inserted to assist the alignment. Double underlined sequences from 301 to 379 is tRNA-glu. The bacterial species are *A. seminis*, *Escherichia coli* and *Aeromonas hydrophila.* The electronic form of sequences of bacteria other than *A. seminis* were kindly supplied by Dr. V. Gurtler.

Appendix 5 cont...



Appendix 6 ***Comparison of rrnB of A. seminis with ribosomal operons of some other bacteria (Gurtler and Stanisich, 1996).*** The sequences are from the end of the 16S gene and the beginning of the 23S gene. Gaps (-) have been inserted to assist the alignment. Double underlined sequences from 222 to 298 and 384 to 460 are tRNA-Ile and tRNA-Ala respectively. The double underlined sequences from 52 to 70 are to show sequence similarity between *S. aureus* and *E. hirae* but this is not a tRNA. The bacterial species are *Aeromonas hydrophila, Escherichia coli, Pseudomonas aeruginosa, Burkholderia ceptica, Staphylococcus aureus, Enterococcus hirae, Rhodobacter sphaeroides, Bacillus subtilis, Clostridium botulinum, Methanococcus vannielii, Streptomyces griseus, Listeria monocytogenes and Mycoplasma hyopneumoniae*. The sequences 1: *A. seminis* spacer region of *rrnB* with two tRNAs and 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 16, 19, 20 are spacer regions with two tRNAs-isoleucine and alanine. The sequences 12, 13 and 14 had only a tRNA-isoleucine. The sequences 17 and 18 had only a tRNA-alanine. The sequences 11, 21, 22, 23, 24, 25, 26, 27 and 28 had no tRNA. The electronic form of sequences of bacteria other than *A. seminis* were kindly supplied by Dr. V. Gurtler.

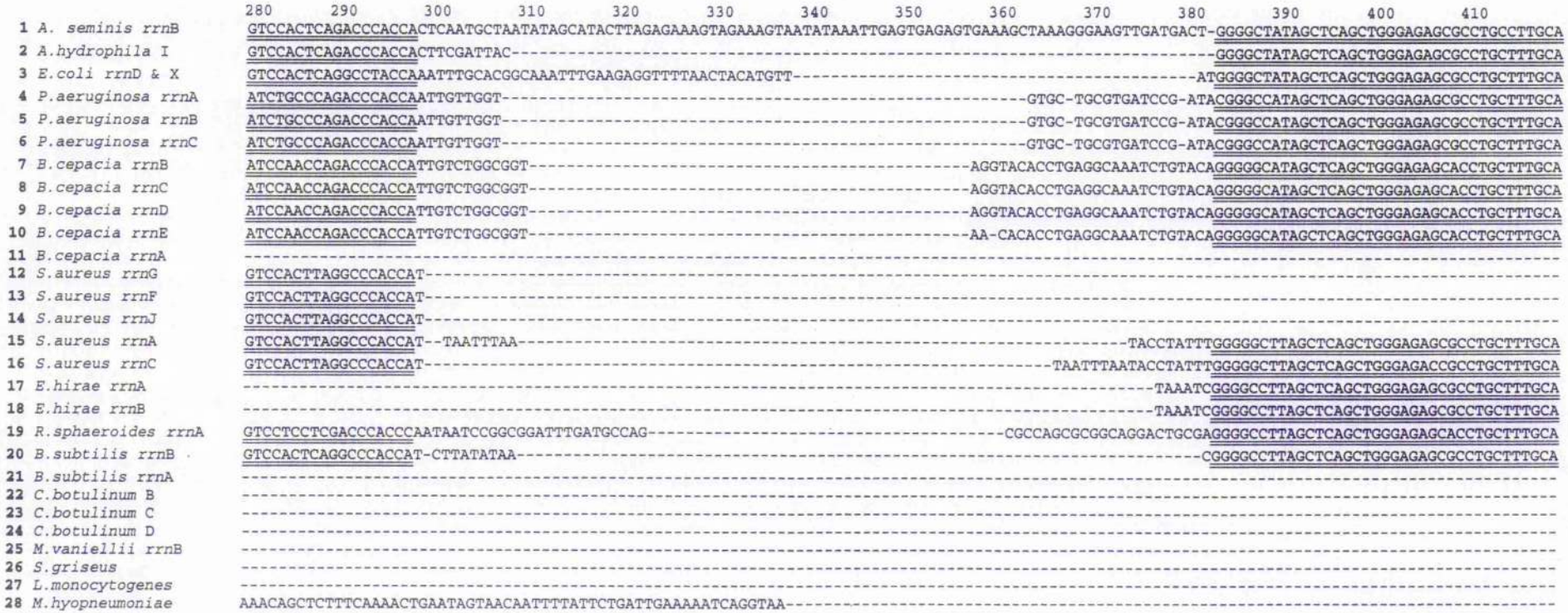
Appendix 6 cont...

Appendix 6 cont...

Appendix 6 cont...

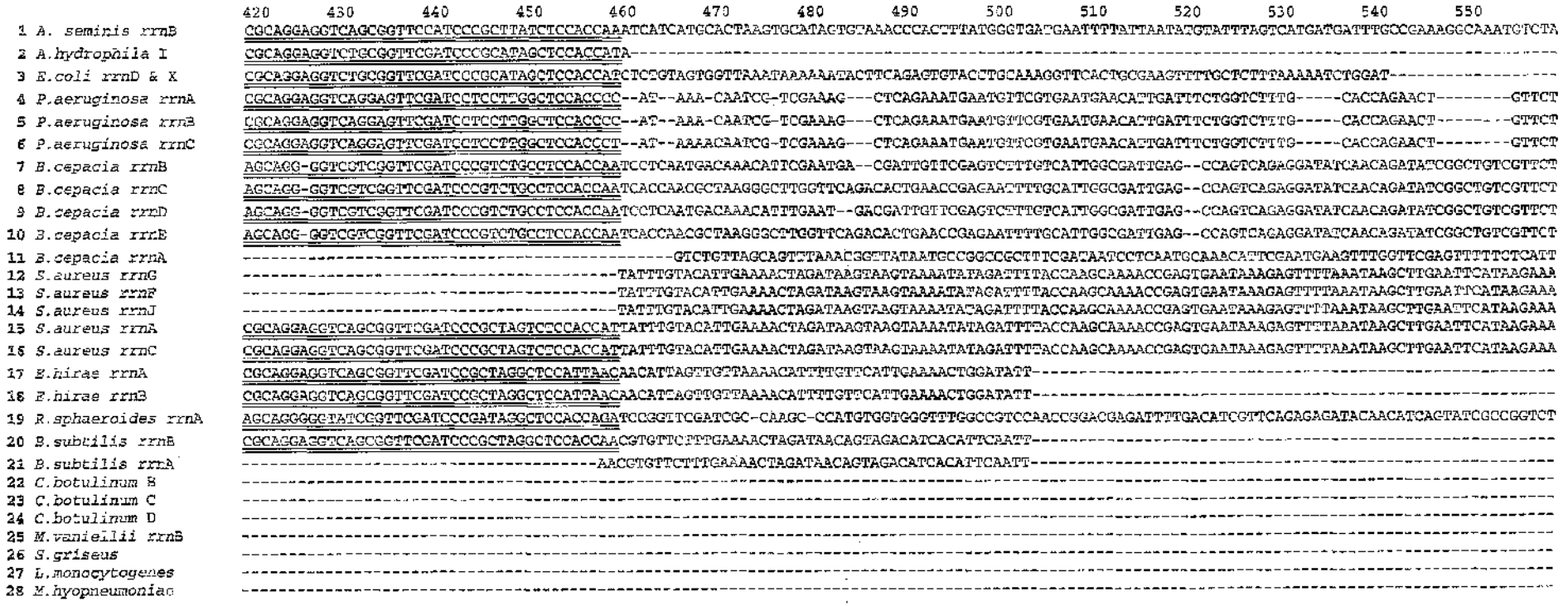
Appendix 6 cont...

Appendix 6 cont...



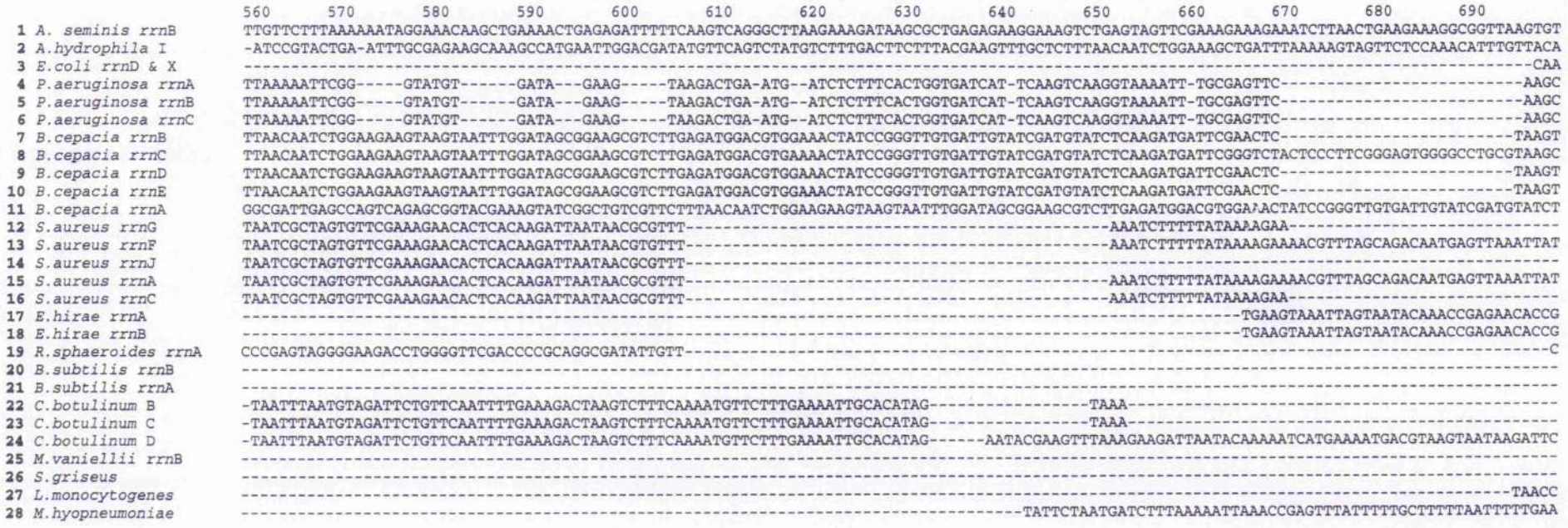
Appendix 6 cont...

Appendix 6 cont...



Appendix 6 cont...

Appendix 6 cont...



Appendix 6 cont...

Appendix 6 cont...

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                    700       710       720       730       740       750       760       770       780       790
 1 A. seminis rrnB  TTAGTTGAAAGATATCGCCTTAAGCATAAAATGCTTGAGGTTGTAT----------------------------------------------------  [604]
 2 A.hydrophila I   AGTGCTTTGGAAACTTGGCGAAAACCAAATTTTATTTGGTCCTTGTTGTACGACAACAAGCTAGCGTGGCTTTCACCGACACTTCTTGGGGTTGTAT  [472]
 3 E.coli rrnD & X  GCTGAAAATTGAAACACTGAACAATGAAAGTTGTTCGTGAGTCTCTCAAATTTTCGCAACTC-----TGAAGTG----AAACATCTTCGGGTTGTGA  [437]
 4 P.aeruginosa rrnA ------------GC-------------GAATT----TTCGGCG-AATGTC-GTCTTCACAGTATAACCA----------GATTGCTTGGGGTTATA-  [467]
 5 P.aeruginosa rrnB ------------GC-------------GAATT----TTCGGCG-AATGTC-GTCTTCACAGTATAACCA----------GATTGCTTGGGGTTATA-  [467]
 6 P.aeruginosa rrnC ------------GC-------------GAATT----TTCGGCG-AATGTC-GTCTTCACAGTATAACCA----------GATTGCTTGGGGTTATA-  [468]
 7 B.cepacia rrnB   ---------TTGAC------------TCAATTGGAATACGGCACAACGCGAGAACTCAACCTGTAACGA----------GACAGACTCGTTATA---  [528]
 8 B.cepacia rrnC   GCTAAAGCGCTAACGCCGGACCGACATCAATTGGAATACGGCACAACGCGAGAACTCAACCTGTAGCGACTGTCGATGAGACAGACTCGTTATA---  [586]
 9 B.cepacia rrnD   ---------TTGAC------------TCAATTGGAATACGGCACAACGCGAGAACTCAACCTGTAACGA----------GACAGACTCGTTATA---  [528]
10 B.cepacia rrnE   ---------TTGAC------------TCAATTGGAATACGGCACAACGCGAGAACTCAACCTGTAACGA----------GACAGACTCGTTATA---  [529]
11 B.cepacia rrnA   CAAGATGATTCGAACTCTAT-GTTCGACTCAATTGGAATACGGCACAACGCGAGAACTCAACCTGTAGCGACTGTCGATGAGACAGACTCGTTATA-  [392]
12 S.aureus rrnG    ----------------------------------------------------CGTAACTTCATGTTAACGTTTGGACTTATAAAAATGGTGGAAACATA-  [382]
13 S.aureus rrnF    TTTAAAGCAG-AGTTTACTTATGTAAATGAGCATTTAAAATAATGAAAACGAAGCCGTATGTGAGCGTTT-GACTTATAAAAATGGTGGAAACATA-  [457]
14 S.aureus rrnJ    ------------------------------------------------------------------------CCTGTAGGA----TGGAAACATA-  [335]
15 S.aureus rrnA    TTTAAAGCAGGAGTTTACTTATGTAAATGAGCATTTAAAATAATGAAAACGAAGCCGTATGTGAGCGTTT-GACTTATAAAAATGGTGGAAACATA-  [551]
16 S.aureus rrnC    ----------------------------------------------------CGTAACTTCATGTTAACGTTT-GACTTATAAAAATGGTGGAAACATA-  [476]
17 E.hirae rrnA     CGTTGAATGAGTTTTTTAATAAGTTCAATTGCTTATTTTCTTGATCGGACTTCTATCGCTAGAAGAAAGATCAAAACCCAACCGTAAGGGTTGATAA  [330]
18 E.hirae rrnB     CGTTGAATGAGTTTTTTAATAAGTTCAATTGCTTATTTTCTTGATCGGACTTCTATCGCTAGAAGAAAGATCAAAACCCAACCGTAAGGGTTGATAA  [330]
19 R.sphaeroides rrnA CAAGTCTAGTACAACTGACCGCGACGACCTTCGGGTCATCGCATGGGAATGTACATGCTTCTGACATGGGAAAGAGCCTTGCTCTTTCCGGATCAG-  [664]
20 B.subtilis rrnB  ------------------------------------------------------------------AGTAACACAAGATATCACATAGTGATTCTTTTAAC  [344]
21 B.subtilis rrnA  ------------------------------------------------------------------AGTAACACAAGATATCACATAGTGATTCTTTTAAC  [167]
22 C.botulinum B    --------------TTGTATATAATACAACAAGCCAAGAATATATTCTTTGTAT--AAATAAGAACTATTCATTAATTGTTAAAATTAAATTAATA-  [366]
23 C.botulinum C    ------------TTGTATATAATACAACAAGCCAAGAAT-TATTCTTTGTATTTAAATAAGAACTATTCATTAATTGTTAAAATTAGATTAATA---  [365]
24 C.botulinum D    AATAAATAAGTATATTGTATATAATACAACAAGCCAAGAATATATTCTTTGTAT--AAATAAGAACTATTCATTAATTGTTAAAATTAAATTAATA-  [437]
25 M.vannielii rrnB ------------------------------------GATATTAATGATGGTCGTGCACAAGCTTTCATATCTGGTGTTATCCAGATGTCTAATTT-  [232]
26 S.griseus        -GCCTTCGACGCCGGCCCCAGTGAACTCGCCTTCAGGGCGGGGTGATGGGTGGCTGGTCGTTGCTTGAGAACTGCACAGTGGACGCGAGCATCTGT-  [304]
27 L.monocytogenes  GAG-AATCATCTGAAAGTGAATCTTTCATCTGATTGGAAGTATCATCGCTGATACGGAAAATCAGAAAAACAACCTTTACTTCGTAGAAGTAAATT-  [243]
28 M.hyopneumoniae  AATTAAAAAATTAAAAAGAAAGATTATGCTAAAATTGTTAAAATACCTTAAGATATATTATCTCAAAATAGGCTATACTCAAAAATAGTTTTAACT-  [491]
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Appendix 7 ***Comparison of sequences of tRNA of different bacterial species.*** The bold letters show differences in the sequences.

Appendix 7 cont...

Genbank accession	Species and strains	Sequence
tRNA-Glu		
AF013275	*A. seminis*	GTCCCCATCGTCTAGAGGCCTAGGACATCGCCCTTTCACGGCGGTAACCGGGGTTCGAATCCCCGTGGG
U07776	*A actinomycetemcomitans* ATCC 29523	GTCCCCATCGTCTAGAGGCCTAGGACATCGCCCTTTCACGGCGGTAACCGGGGTTCGAATCCCCGTGGG
U07778	*A.actinomycetemcomitans* ATCC 29522	GTCCCCATCGTCTAGAGGCCTAGGACATCGCTCTTTCACGGCGGTAACCGGGGTTCGAATCCCCGTGGG
U87825	*H. ducreyi*	GTCCCCATCGTCTAGAGGCCTAGGACATCGCCCTTTCACGGCGGTAACCGGGGTTCGAATCCCCGTGGG
U32745	*H. influenzae* from bases 658092 to 668483	GTCCCCATCGTCTAGAGGCCTAGGACATCGCCCTTTCACGGCGGTAACCGGGGTTCGAATCCCCGTGGG
K00188	*E.coli* Glu-tRNA-1	GTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTTTCACGGCGGTAACAGGGGTTCGAATCCCCTGGGG
M25658	*E.coli* complete transfer RNA-Glu2	GTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTNTCACGGCGGTAACAGGGGTTCGAATCCCCTAGGG
X65486	*P.shigelloides*	GTCCCCTTCGTCTAGAGGCCTAGGACACCGCCCTTTCACGGCGGTAACAGGGGTTCGAATCCCCTAGGG
L25601	*Aeromonas salmonicida*	GTCCCCTTCGTCTAGAGGCCTAGGACACCGCCCTTTCACGGCGGTAACAGGGGTTCGAATCCCCTAGGG
U10499	*Buchnera aphidicola*	GTCCCCTTCGTCTAGAGGACTAGGACATCGCCCTTTCACGGCGGTAACAGGGGTTCAAATCCCCTAGGG
tRNA-Ile		
AF013276	*A. seminis*	GGGTCTGTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCAAGTCCACTCAGACCCACCA
U32755	*H. influenzae* from bases 766814 to 777380	GGGTCTGTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCAAGTCCACTCAGACCCACCA
K00217	*E.coli* Ile-tRNA-1	GGCT-TGTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCAAGTCCACTCAGGCCTACCA
K00763	*E.coli* rRNA operon (rrnX)	GGCT-TGTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCAAGTCCACTCAGGCCTACCA
U26683	*Dichelobacter nodosus*	GGGTCTGTAGCTCAGTTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCAGAGGTTCAAGTCCTCTCAGACCCACCA
AB003402	*Prevotella ruminicola*	GGCT-TGTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCAAGTCCACTCAGGCCTACCA
J01551	*B.subtilis* ile-tRNA	GGGCCTGTAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGGTGGTTCGAGTCCACTCAGGCCCACCA
K01390	*B.subtilis* 168 trrnE gene cluster	GGGCCTGTAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCATTCAGGCCCACCA
X06693	*P. seudomonas* aeruginosa	GGGTCTGTAGCTCAGTTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGCAGTTCGAATCTGCCCAGACCCACCA
D87087	*P. seudomonas* corrugata	GGGTCTGTAGCTCAGTTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGCAGTTCGAATCTGCCCAGACCCACCA
U11789	*S. aureus*	GGGCCTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGGTGGTTCGAGTCCACTTAGGCCCACCA
tRNA-Ala		
AF013276	*A. seminis*	GGGATATAGCTTAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCCATCCCGCTTATCTCCACCAA
U32755	*H. influenzae* from bases 766814 to 777380	GGGATATAGCTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTATCTCCACCA-
AB003402	*Prevotella ruminicola*	GGGCTATAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCTGCGGTTCGATCCCGCATAGCTCCACCA-
K00763	*E.coli* rRNA operon (rrnX)	GGGCTATAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCTGCGGTTCGATCCCGCATAGCTCCACCA-
D12649	*E.coli* rrnH gene	GGGCTATAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCTGCGGTTCGATCCCGCATAGCTCCACCA-
L28159	*Pseudomonas mendocina*	GGGCCATAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCACCA-
U65012	*Pseudomonas stutzeri*	GGGCCATAGCTCAGCTGGGAGAGGGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCACCA-
D86357	*Pseudomonas syringae*	GGGCCATAGCTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCACCA-
Z29326	*C.jejuni* TGH9011(ATCC43431)	GGGAATTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTATTCTCCACCA-
U11786	*Staphylococcus aureus*	GGGGCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTAGTCTCCACCA-
J01551	*B.subtilis*	GGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTAGGCTCCACCAA
U57918	*Listeria grayi*	GGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTAGGCTCCACCAA
U57913	*Listeria ivanovii*	GGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTAGGCTCCACCAA
U44061	*Listeria monocytogenes*	GGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTAGGCTCCACCAA
U57915	*Listeria innocua*	GGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTAGGCTCCACCAA