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PHENOTYPIC AND MOLECULAR CHARACTERIZATIONS OF STREPTOCOCCUS SPP. ISOLATED FROM BOVINE MAMMARY GLANDS

MD. FIROZ MIAN

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STREPTOCOCCUS SPP. ISOLATED FROM BOVINE MAMMARY GLANDS

By

MD. FIROZ MIAN

Thesis Submitted in Fulfilment of the Requirement for the Degree of
Doctor of Philosophy in the Faculty of Veterinary Medicine
Universiti Putra Malaysia

October 2001
DEDICATION

TO THE MEMORY OF MY BEREAVED FATHER AND TO MY MOTHER
TO MY WIFE MASUDA AND DAUGHTER FARHIN
TO MY BROTHER GULAM MUSTAFA

AND

TO LATE Dr. M. FAZLUR RAHMAN, CHIEF SCIENTIFIC OFFICER, BLRI
Abstract of the thesis presented to the Senate of the Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PHENOTYPIC AND MOLECULAR CHARACTERIZATIONS OF STREPTOCOCCUS SPP. ISOLATED FROM BOVINE MAMMARY GLANDS

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October 2001

Chairman: Abdul Rahim Mutilib, DVM, MS, Ph.D.

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Sixty-two streptococcal isolates comprising 20 Streptococcus agalactiae, 18 S. dysgalactiae and 24 S. uberis isolates were recovered from clinical and subclinical cases of bovine mastitis from different dairy herds in the Selangor state in Malaysia. A simple biochemical test scheme formulated on the basis of seven biochemical reactions allowed the identification of S. agalactiae, S. dysgalactiae and S. uberis isolates within 24 hours. Streptococcus agalactiae isolates were β-haemolytic, CAMP positive, utilized hippurate, salicin and raffinose; S. dysgalactiae isolates were α-haemolytic and fermented only trehalose and raffinose, while S. uberis isolates showed positive reactions to esculin, inulin and mannitol. The API 20 Strep System characterized accurately 100% of S. agalactiae and S. dysgalactiae isolates, and 96.1% of S. uberis isolates although some variable reactions among the isolates within the species were observed. Majority of the isolates were susceptible to most of the antimicrobial agents tested. All S. agalactiae, S. dysgalactiae and S. uberis isolates were susceptible to oxacillin and nitrofurantoin, while 30% of the isolates were resistant to tetracycline. Most of the S. dysgalactiae isolates showed resistance
to kanamycin and cephalexin, and *S. agalactiae* isolates to kanamycin. *Streptococcus dysgalactiae* isolates showed higher level of resistance compared to *S. agalactiae* and *S. uberis*. Serotyping of the streptococcal isolates using monospecific antisera in agar gel double immunodiffusion revealed that all *S. agalactiae* isolates were typeable and demonstrated the type patterns II (60%), Ia (20%), III (15%) and IV (5%). On the other hand, 17 (94.4%) *S. dysgalactiae* and 22 (91.6%) *S. uberis* isolates were also identified.

The SDS-PAGE and Western-blotting analyses revealed antigenic heterogeneity among the isolates of the bovine *Streptococcus* species examined. Sodium dodecylsulphate polyacrylamide gel electrophoresis could differentiate the three streptococcal species on the basis of their characteristic polypeptide bands. The Western blot analysis also revealed obvious differences in immunogenic proteins between the streptococcal species. Moreover, isolates within each species produced variable protein bands on PAGE analysis and variable immunogenic proteins by Western blotting which let the basis to group them into distinct PAGE and immunoblot fingerprint profiles respectively.

Random amplified polymorphic DNA (RAPD) analysis was evaluated for its capacity to distinguish strains within the species of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* and for epidemiological subtyping. Three single primers were used for each species to generate characteristic RAPD fingerprints. The DNA fingerprint patterns obtained with each primer were distinct and reproducible. The RAPD fingerprints generated could be useful in delineating the strains of *S. agalactiae*, *S. dysgalactiae*
and *S. uberis*. The intraspecies typing efficiency was significantly improved by the parallel use of three primers. The RAPD results showed high level of genetic diversity within strains of the streptococcal species.

The amplification of the DNA encoding 16S rRNA genes by polymerase chain reaction with single set of primers complementary to 16S rRNA gene regions generated characteristic single amplicon that enabled identification and differentiation of the three streptococcal species. The restriction fragment length polymorphism (RFLP) analysis of the amplified 16S rRNA gene regions with the restriction enzymes *MspI* and *RsaI* produced reproducible fingerprint patterns indicating high level of genetic diversity among the isolates of the streptococcal species. Higher heterogeneity was observed within *S. uberis* and *S. dysgalactiae* isolates than the *S. agalactiae* isolates. The discriminatory powers of the two enzymes were to some extent similar.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN FENOTIP DAN MOLEKUL STREPTOCOCCUS SPP. YANG DIPENCIL DARIPADA KELENJAR MAMA BOVIN

Oleh

MD. FIROZ MIAN

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Enam puluh dua pencilan streptokokus terdiri daripada 20 Streptococcus agalactiae, 18 S. dysgalactiae dan 24 S. uberis telah diperolehi daripada kes klinikal dan subklinikal mastitis bovin berbagai kelompok lembu tenusu dalam negeri Selangor, Malaysia. Suatu skema ujian biokimia mudah yang dirumus berasaskan tujuh tindak balas, telah membolehkan pengenalpastian pencilan S. agalactiae, S. dysgalactiae, dan S. uberis dalam tempoh 24 jam. Pencilan Streptococcus agalactiae ialah α-hemolisis, positif pada ujian CAMP, pengguna hipurat, salisin dan rafinosa; pencilan S. dysgalactiae ialah α-hemolisis dan hanya menapai trehalosa dan rafinosa, sambil S. uberis menunjukkan tindak balas positif terhadap eskulin, inulin, dan manitol. API 20 Strep System mencirikan dengan tepat 100% daripada pencilan S. agalactiae dan S. dysgalactiae, 96.1% daripada pencilan S. uberis, walaupun terdapat pelbagai tindak balas di kalangan pencilan dalam sesuatu spesies. Sebahagian besar daripada pencilan ini rentan terhadap kebanyakan agen antimikrob yang diuji.
Kesemua pencilan *S. agalactiae*, *S. dysgalactiae* dan *S. uberis* adalah rentan terhadap oksasilin dan nitrofurantoin, sambil 30% daripada pencilan tahan tetrasiiklin. Kebanyakan daripada pencilan *S. dysgalactiae* menunjukkan ketahanan terhadap kanamisin dan sefaleksin, dan pencilan *S. agalactiae* pula terhadap kanamisin. Pencilan *Streptococcus dysgalactiae* menunjukkan ketahanan lebih tinggi berbanding *S. agalactiae* atau *S. uberis*. Penserotipan pencilan streptokokus menggunakan antiserum monokhusus dalam gel agar-agar pengimunosap dedua menunjukkan pencilan *S. agalactiae* boleh ditip dan menyatakan pola tip II (60%), III (20%), dan IV (5%). Disebaliknya, 17 (94.4%) pencilan *S. dysgalactiae* dan 22 (91.6%) pencilan *S. uberis* telah dikenal pasti.

Analisis SDS-PAGE dan pensapan Western menunjukkan keheterogenan antigen di kalangan pencilan spesies *Streptococcus* bovin yang dikaji. Elektroforesis gel poliakrilamida natrium dodesilsulfat boleh membeza tiga spesies streptokokus berasaskan jalur polipeptida cirian. Analisis sap Western juga menunjukkan kelainan nyata dalam protein imunogen di antara spesies streptokokus. Tambahan pula pencilan di dalam setiap spesies menghasilkan jalur protein pelbagai pada analisis PAGE dan protein imunogen pelbagai pada pengsapan Western, yang mengesahkan asas untuk mengumpulkan pencilan ini masing-masing kepada profil sidikjari PAGE dan imunosap.

Analisis DNA polimorfik terkuat rawak (RAPD) telah dinilaikan untuk keupayaannya membeza strain di kalangan spesies *S. agalactiae*, *S. dysgalactiae* dan *S. uberis* dan untuk pengsubtipan epidemiologi. Pola sidikjari DNA yang diperolehi daripada setiap primer adalah jelas dan boleh dihasil semula. Sidikjari RAPD yang
dijanakan mungkin berguna dalam membeza di antara strain *S. agalactiae*, *S. dysgalactiae* dan *S. uberis*. Kecekapan pengetipan intraspesies nyata lebih baik dengan penggunaan tiga primer. Hasil RAPD menunjukkan yang aras kepelbagaian genetik di kalangan strain spesies streptokokus adalah tinggi.

Penguatan gen 16S rRNA pengekod DNA melalui tindak balas rangkaian polimerase menggunakan satu set primer pelengkap kepada kawasan gen 16S RNA menjanakan amplikon tunggal cirian yang membolehkan untuk pengenalpastian dan pembezaan tiga spesies streptokokus dilakukan. Analisis polimorfosime panjang fragmen pengehadan (RFLP) terhadap kawasan gen 16S RNA terkuat dengan menggunakan enzim pengehadan *MspI* dan *RsaI* menghasilkan pola sidikjari boleh dihasil semula, menunjukkan yang adanya kepelbagaian genetik aras tinggi di kalangan pencilan spesies streptokokus. Keheterogenan lebih tinggi telah dicerap di kalangan pencilan *S. uberis* dan *S. dysgalactiae* berbanding pencilan *S. agalactiae*. Kuasa pembezaan untuk dua enzim ini agak sama.
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I certify that an Examination Committee met on 13th October 2001 to conduct the final examination of Md. Firoz Mian on his Doctor of Philosophy thesis entitled “Phenotypic and Molecular Characterizations of Streptococcus spp. Isolated from Bovine Mammary Glands” in accordance with Universiti Pertanian Malaysia (Higher degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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Date: 15 DEC 2001
I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at Universiti Putra Malaysia or other institutions.

Date: 01-11-2001

MD. FIROZ MIAN
TABLE OF CONTENTS

DEDICATION ........................................................................................................... ii
ABSTRACT ............................................................................................................... iii
ABSTRAK ............................................................................................................... xi
ACKNOWLEDGEMENTS ....................................................................................... vi
APPROVAL SHEETS ............................................................................................... xi
DECLARATION FORM ............................................................................................ xiii
LIST OF TABLES ...................................................................................................... xv
LIST OF FIGURES .................................................................................................. xx
LIST OF ABBREVIATIONS ...................................................................................... xxiv

CHAPTER

I

INTRODUCTION ................................................................................................. 1

II

LITERATURE REVIEW ......................................................................................... 9
Streptococci ........................................................................................................ 9
  Streptococcus agalactiae .................................................................................. 10
  Streptococcus dysgalactiae .......................................................................... 11
  Streptococcus uberis ................................................................................... 11
Bovine Streptococcal Mastitis ........................................................................ 12
Bacterial Isolation and Identification .............................................................. 14
Isolation and Culture Media ............................................................................. 14
Identification ...................................................................................................... 16
Serological Characterization .......................................................................... 19
Molecular Characterization of Streptococci .................................................... 22
DNA-based Molecular Techniques ................................................................ 22
  Random Amplified Polymorphic DNA-PCR .............................................. 23
  Restriction Enzyme Analysis ..................................................................... 25
  DNA Hybridizations .................................................................................... 26
  Analysis of 16S rRNA gene ....................................................................... 27
  Pulsed-Field Gel Electrophoresis ............................................................... 29
Protein-based Molecular Techniques ............................................................. 30
  Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting ........................................... 30
Antimicrobial Sensitivity ............................................................................... 33
Virulence Factors and Pathogenicity ............................................................... 35
Pathogenesis ..................................................................................................... 37
Epidemiology ...................................................................................................... 40
Clinical and Pathological Manifestations ....................................................... 44
  Clinical Manifestations ............................................................................. 44
  Pathological Changes ............................................................................... 45
Diagnosis ............................................................................................................ 46
  Clinical Examination and Detection using a strip cup ................................ 46
Western Immunoblotting ........................................... 93
Protein Transfer to the Nitrocellulose Membrane ............. 94
Immunodetection of the Blotted Proteins ....................... 95
Results ........................................................................... 96
PAGE-Protein Patterns ............................................... 96
Western Immunoblotting ........................................... 104
Discussion ....................................................................... 112

V
MOLECULAR CHARACTERIZATION OF
STREPTOCOCCUS SPP. ISOLATED FROM BOVINE
MAMMARY GLANDS BY RANDOM AMPLIFIED
POLYMORPHIC DNA-PCR ANALYSIS ......................... 123
Introduction ..................................................................... 123
Materials and Methods ................................................ 127
Streptococcal isolates ................................................... 127
Genomic DNA Preparation ........................................... 127
Determination of DNA Concentration ......................... 128
RAPD-PCR Primers ...................................................... 129
RAPD-PCR Conditions ................................................ 129
Agarose Gel Electrophoresis ......................................... 130
Results ........................................................................... 130
Discussion ....................................................................... 148

VI
MOLECULAR CHARACTERIZATION OF
STREPTOCOCCUS SPP. FROM BOVINE UDDEN
INFECTIONS BY ANALYSIS OF 16S rRNA GENE ............ 154
Introduction ..................................................................... 154
Materials and Methods ................................................ 158
Bacterial isolates ......................................................... 158
Isolation of Bacterial DNA ........................................... 158
PCR Amplification of 16S rRNA gene ............................. 159
DNA Primers ............................................................... 159
PCR Conditions ........................................................... 159
Gel Electrophoresis ...................................................... 160
Direct Purification of Amplified 16S rRNA gene ............. 160
Enzymatic Digestions ................................................... 161
Agarose Gel Electrophoresis ......................................... 162
16S rRNA gene-RFLP Analysis .................................... 162
Results ........................................................................... 163
Amplification of 16S rRNA gene by PCR ....................... 163
RFLP Analysis of 16S rRNA gene ................................. 163
Discussion ..................................................................... 176

VII
GENERAL DISCUSSION .................................................. 182

BIBLIOGRAPHY ...........................................................
APPENDICES .............................................................................. 234
  Appendix A: Reagents and Preparations for
  Bacteriological Techniques ................................................ 235
  Appendix B: SDS-PAGE Buffers and Solutions ............... 239
  Appendix C: Western Blotting Buffers and Reagents ...... 245
  Appendix D: Reagents and Buffers for DNA Analysis .... 248

BIO-DATA OF THE AUTHOR ...................................................... 252
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Streptococcal isolates collected from bovine mammary glands from different dairy herds in Selangor, Malaysia.</td>
<td>71</td>
</tr>
<tr>
<td>3.1</td>
<td>Conventional biochemical test scheme for characterization of <em>Streptococcus agalactiae</em>, <em>S. dysgalactiae</em> and <em>S. uberis</em> isolates from bovine intramammary infections.</td>
<td>73</td>
</tr>
<tr>
<td>3.2</td>
<td>Biochemical characteristics (% positive) of <em>Streptococcus agalactiae</em>, <em>S. dysgalactiae</em> and <em>S. uberis</em> isolates utilizing API 20 Strep System.</td>
<td>74</td>
</tr>
<tr>
<td>3.3</td>
<td>Antimicrobial susceptibility patterns of <em>Streptococcus agalactiae</em>, <em>S. dysgalactiae</em> and <em>S. uberis</em> isolated from bovine IMI.</td>
<td>76</td>
</tr>
<tr>
<td>3.4</td>
<td>Serotype distributions of <em>S. agalactiae</em>, <em>S. dysgalactiae</em> and <em>S. uberis</em> isolates recovered from bovine mastitis.</td>
<td>78</td>
</tr>
<tr>
<td>4.1</td>
<td>Number and molecular weights of <em>Streptococcus agalactiae</em> polypeptide bands generated by 12% PAGE stained with coomassie blue.</td>
<td>98</td>
</tr>
<tr>
<td>4.2</td>
<td>Number and molecular weights of <em>Streptococcus dysgalactiae</em> polypeptide bands generated by 12% PAGE stained with coomassie blue.</td>
<td>99</td>
</tr>
<tr>
<td>4.3</td>
<td>Number and molecular weights of <em>Streptococcus uberis</em> polypeptide bands generated by 12% PAGE stained with coomassie blue.</td>
<td>100</td>
</tr>
<tr>
<td>5.1</td>
<td>The differences among <em>Streptococcus agalactiae</em> isolates in blotted polypeptide bands immunologically detected by rabbit hyperimmune serum to <em>S. agalactiae</em>.</td>
<td>106</td>
</tr>
<tr>
<td>5.2</td>
<td>The differences among <em>Streptococcus dysgalactiae</em> isolates in blotted polypeptide bands immunologically detected with rabbit hyperimmune serum to <em>S. dysgalactiae</em>.</td>
<td>107</td>
</tr>
<tr>
<td>5.3</td>
<td>The differences among <em>Streptococcus uberis</em> isolates in blotted polypeptide bands immunologically detected with rabbit hyperimmune serum to <em>S. uberis</em>.</td>
<td>108</td>
</tr>
</tbody>
</table>

xviii
6.1 RAPD fingerprint profiles among the *S. agalactiae* isolates generated with primer OPA-01, OPA-07 and OPA-13

6.2 RAPD fingerprint profiles among the *Streptococcus dysgalactiae* isolates generated with primers OPA-01, OPA-07 and OPA-13

6.3 RAPD fingerprinting profiles of *Streptococcus uberis* isolates generated with primers OPA-01, OPA-05 and OPA-07
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Double immunodiffusion reactions between antisera against serotype II and hot acid extracts of <em>Streptococcus agalactiae</em> isolates (A), and extracts of <em>S. uberis</em> isolates and specific antisera against the reference strain 0140J (B) (two isolates did not react)</td>
<td>77</td>
</tr>
<tr>
<td>4.1</td>
<td>SDS-PAGE protein patterns of <em>Streptococcus agalactiae</em> isolates separated on 12% polyacrylamide gels and stained with coomassie brilliant blue; Lane M: broad range protein molecular standard (kDa)</td>
<td>101</td>
</tr>
<tr>
<td>4.2</td>
<td>SDS-PAGE protein patterns of <em>Streptococcus dysgalactiae</em> isolates separated on 12% polyacrylamide gels and stained with coomassie brilliant blue; Lane M: broad range protein molecular standard (kDa)</td>
<td>102</td>
</tr>
<tr>
<td>4.3</td>
<td>SDS-PAGE protein patterns of <em>Streptococcus uberis</em> isolates separated on 12% polyacrylamide gels and stained with coomassie brilliant blue; Lane M: broad range protein molecular standard (kDa)</td>
<td>103</td>
</tr>
<tr>
<td>5.1</td>
<td>Western immunoblots of whole-cell antigens of <em>Streptococcus agalactiae</em> isolates electrophoresed on 12% SDS-PAGE gels, electrotransferred to nitrocellulose membranes and reacted with rabbit hyperimmune serum to <em>S. agalactiae</em>; Lane M: broad range protein molecular marker (kDa)</td>
<td>109</td>
</tr>
<tr>
<td>5.2</td>
<td>Western immunoblots of whole-cell antigens of <em>Streptococcus dysgalactiae</em> isolates electrophoresed on 12% SDS-PAGE gels, electrotransferred to nitrocellulose membranes and reacted with rabbit hyperimmune serum to <em>S. dysgalactiae</em>; Lane M: broad range protein molecular marker (kDa)</td>
<td>110</td>
</tr>
<tr>
<td>5.3</td>
<td>Western immunoblots of whole-cell antigens of <em>Streptococcus uberis</em> isolates electrophoresed on 12% SDS-PAGE gels, electrotransferred to nitrocellulose membranes and reacted with rabbit hyperimmune serum to <em>S. uberis</em>; Lane M: broad range protein molecular marker (kDa)</td>
<td>111</td>
</tr>
<tr>
<td>6.1</td>
<td>RAPD fingerprinting patterns of <em>Streptococcus agalactiae</em> isolates generated with primer OPA-01 and electrophoresed in 1% agarose gel; Lane M: 1 Kb DNA ladder</td>
<td>134</td>
</tr>
</tbody>
</table>
6.2 RAPD fingerprinting patterns of *Streptococcus agalactiae* isolates generated with primer OPA-07 and electrophoresed in 1% agarose gel; Lane M: 1 Kb DNA ladder

6.3 RAPD fingerprinting patterns of *Streptococcus agalactiae* isolates generated with primer OPA-13 and electrophoresed in 1% agarose gel; Lane M: 1 Kb DNA ladder

6.4 Dendogram of the cluster analysis based on the RAPD fingerprint patterns of *Streptococcus agalactiae* isolates generated with primers OPA-01, OPA-07 and OPA-13

6.5 RAPD fingerprinting patterns of *Streptococcus dysgalactiae* isolates generated with primer OPA-01 and electrophoresed in 1% agarose gel; Lane M: 1 Kb DNA ladder

6.6 RAPD fingerprinting patterns of *Streptococcus dysgalactiae* isolates generated with primer OPA-07 and electrophoresed in 1% agarose gel; Lane M: 1 Kb DNA ladder

6.7 RAPD fingerprinting patterns of *Streptococcus dysgalactiae* isolates generated with primer OPA-13 and electrophoresed in 1% agarose gel; Lane M: 1 Kb DNA ladder

6.8 Dendogram of the cluster analysis based on the RAPD fingerprint patterns of *Streptococcus dysgalactiae* isolates generated with primers OPA-01, OPA-07 and OPA-13

6.9 RAPD fingerprinting patterns of *Streptococcus uberis* isolates generated with primer OPA-01 and electrophoresed in 1% agarose gel; Lane M: 1 Kb DNA ladder

6.10 RAPD fingerprinting patterns of *Streptococcus uberis* isolates generated with primer OPA-07 and electrophoresed in 1% agarose gel; Lane M: 1 Kb DNA ladder

6.11 RAPD fingerprinting patterns of *Streptococcus uberis* isolates generated with primer OPA-05 and electrophoresed in 1% agarose gel; Lane M: 1 Kb DNA ladder

6.12 Dendogram of the cluster analysis based on the RAPD fingerprint patterns of *Streptococcus uberis* isolates generated with primers OPA-01, OPA-07 and OPA-05
7.1 The fragments of the PCR amplified 16S rRNA genes of *Streptococcus agalactiae* (A), *S. dysgalactiae* (B) and *S. uberis* (C); Lane M: 100 bp plus DNA molecular size marker.

7.2 Schematic representation of PCR amplified 16S rRNA gene-RFLP patterns of *Streptococcus agalactiae* isolates generated by digestions with *MspI* and *RsaI*; Lane M: 100 bp plus DNA molecular size marker.

7.3 The 16S rRNA gene-RFLP fingerprint profiles of *Streptococcus agalactiae* isolates produced upon digestion with *MspI* and electrophoresed in 2% agarose gels; Lane M: 100 bp plus DNA molecular size marker.

7.4 The 16S rRNA gene-RFLP fingerprint profiles of *Streptococcus agalactiae* isolates produced upon digestion with *RsaI* and electrophoresed in 2% agarose gels; Lane M: 100 bp plus DNA molecular size marker.

7.5 Schematic representation of PCR amplified 16S rRNA gene-RFLP patterns of *Streptococcus dysgalactiae* isolates generated by digestions with *MspI* and *RsaI*; Lane M: 100 bp plus DNA molecular size marker.

7.6 The 16S rRNA gene-RFLP fingerprint profiles of *Streptococcus dysgalactiae* isolates produced upon digestion with *MspI* and electrophoresed in 2% agarose gels; Lane M: 100 bp plus DNA molecular size marker.

7.7 The 16S rRNA gene-RFLP fingerprint profiles of *Streptococcus dysgalactiae* isolates produced upon digestion with *RsaI* and electrophoresed in 2% agarose gels; Lane M: 100 bp plus DNA molecular size marker.

7.8 Schematic representation of PCR amplified 16S rRNA gene-RFLP patterns of *Streptococcus uberis* isolates generated by digestions with *MspI* and *RsaI*; Lane M: 100 bp plus DNA molecular size marker.

7.9 The 16S rRNA gene-RFLP fingerprint profiles of *Streptococcus uberis* isolates produced upon digestion with *MspI* and electrophoresed in 2% agarose gels; Lane M: 100 bp plus DNA molecular size marker.

xxii
7.10 The 16S rRNA gene-RFLP fingerprint profiles of *Streptococcus uberis* isolates produced upon digestion with *RsaI* and electrophoresed in 2% agarose gels; Lane M: 100 bp plus DNA molecular size marker.
LIST OF ABBREVIATIONS

Ab    Antibody
AP-PCR Arbitrary Primed-Polymerase Chain Reaction
bp    Base pair
BSA   Bovine serum albumin
°C    Degree Celcius
cfu   Colony forming unit
cm    Centimeter
CMT   California Mastitis Test
DNA   Deoxyribonucleic acid
dNTP  Deoxy-nucleotide triphosphate
Dr.   Doctor
e.g.  For example
EDTA  Ethylene Diamine tetra-acetate
ELISA Enzyme Linked Immunosorbent Assay
g    Gram
g/l   Gram per litre
G+C   Guanine + Cytosine
H₂O₂  Hydrogen peroxide
h/hrs Hour/Hours
HIS   Hyperimmune Serum
i.e.  That is
i.m.  Intramuscular
IgA   Immunoglobulin A
IgG   Immunoglobulin G
Kbp   Kilobase pairs
kDa   Kilodalton
M     Molar
Mab   Monoclonal Antibody
M.W.  Molecular Weight
Mg    Milligram
min/mins Minute/Minutes
ml    Millilitre
mM    Millimolar
nm    Nanometer
O.D.  Optical Density
PAGE  Polyacrylamide Gel Electrophoresis
PBS   Phosphate Buffer Saline
PBST  Phosphate Buffer Saline Tween 20
PCR   Polymerase Chain Reaction
pH    Hydrogen ion concentration
PFGE  Pulsed Field Gel Electrophoresis

xxiv