PASTEURELLA AND MANNHEIMIA SPECIES FROM CALVES:  
DIFFERENTIATION AND ANTIMICROBIAL RESISTANCE

BOUDEWIJN CATRY

MERELBEKE, 2005
Front cover: Macroscopic detail of *Pasteurella multocida* on Columbia sheep blood agar.

Back cover: Picture taken by Catry Frederick, 2001.


**Suggested citation:** Catry B. (2005). *Pasteurella* and *Mannheimia* species from calves: differentiation and antimicrobial resistance, PhD thesis, Ghent University, Merelbeke, Belgium.

**ISBN:** 90-5864-082-5

**EAN:** 9789058640826
PASTEURELLA AND MANNHEIMIA SPECIES FROM CALVES: DIFFERENTIATION AND ANTIMICROBIAL RESISTANCE

BOUDEWIJN CATRY

THESIS TO OBTAIN THE ACADEMIC DEGREE OF DOCTOR OF VETERINARY SCIENCE (PhD)

PROMOTERS:
PROF. DR. DR. H.C. A. DE KRUIF A
PROF. DR. F. HAESEBROUCK B

CoPROMOTER:
PROF. DR. G. OPSOMER A

A DEPARTMENT OF REPRODUCTION, OBSTETRICS, AND HERD HEALTH
B DEPARTMENT OF PATHOLOGY, BACTERIOLOGY AND POULTRY DISEASES
FACULTY OF VETERINARY MEDICINE
GHENT UNIVERSITY, B-9820 MERELBEKE, BELGIUM.

MERELBEKE, 2005
poor is The pupil who does not surpass his master.

Leonardo Da Vinci
# TABLE OF CONTENTS

*Abbreviation key*  
1  
**Preface**  
3  

## Chapter 1. General Introduction  
5  
1.1. Review of literature  
7  
1.1.1. Classification and characteristics of *Pasteurellaceae* associated with bovine disease  
7  
1.1.2. Molecular identification of *Pasteurellaceae*  
12  
1.1.3. Definition and diagnosis of bovine pasteurellosis  
15  
1.1.4. Antimicrobial therapy and resistance monitoring for bovine pasteurellosis  
17  
1.1.5. Fundamentals of antimicrobial resistance in relation to bovine pasteurellosis  
21  
1.2. Aims of the Study  
41  

## Chapter 2. Experimental & Observational Studies  
43  
2.1. tRNA-intergenic spacer PCR for the identification of *Pasteurella* and *Mannheimia* spp.  
45  
2.2. Antimicrobial resistance in *Pasteurella* and *Mannheimia* spp. of calves  
65  
2.2.1. Detection of tetracycline resistant and susceptible *Pasteurellaceae* in the nasopharynx of loose group housed calves  
67  
2.2.2. Variability in acquired resistance of *Pasteurella* and *Mannheimia* isolates from the nasopharynx of calves with particular reference to different herd types  
81  
2.2.3. *tet*(L)-mediated tetracycline resistance in bovine *Pasteurella* and *Mannheimia* isolates  
101  
2.2.4. Fatal peritonitis in calves caused by a multi-resistant *P. multocida* capsular type F  
113  
2.2.5. Novel spectinomycin/streptomycin resistance gene, *aadA14*, from *P. multocida*  
125  

## Chapter 3. General Discussion  
137  

**Summary-Samenvatting**  
153  
**Curriculum Vitae**  
169  
**Concluding Remarks**  
175
### Abbreviation key

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAD</td>
<td>adenyltransferase</td>
</tr>
<tr>
<td>AFLP</td>
<td>amplified fragment length polymorphism</td>
</tr>
<tr>
<td>AMGP</td>
<td>antimicrobial growth promoter</td>
</tr>
<tr>
<td>ANT</td>
<td>nucleotidyltransferase</td>
</tr>
<tr>
<td>AP</td>
<td>arbitrary primed</td>
</tr>
<tr>
<td>ARI</td>
<td>antimicrobial resistance index</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>AUCI</td>
<td>area under the inhibitory curve</td>
</tr>
<tr>
<td>BAC</td>
<td>bacitracin</td>
</tr>
<tr>
<td>BAPOCC</td>
<td>Belgian Antibiotic Policy Coordination Committee</td>
</tr>
<tr>
<td>BEB</td>
<td>bovine enzootic bronchopneumonia</td>
</tr>
<tr>
<td>BPI3</td>
<td>bovine parainfluenza virus type 3</td>
</tr>
<tr>
<td>BRD</td>
<td>bovine respiratory disease</td>
</tr>
<tr>
<td>BRSV</td>
<td>bovine respiratory syncytial virus</td>
</tr>
<tr>
<td>BVD(V)</td>
<td>bovine viral diarrhoea virus</td>
</tr>
<tr>
<td>CCCG</td>
<td>Culture Collection University of Göteborg (S)</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum tissue/blood concentration</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxy-ribonucleic acid</td>
</tr>
<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii (Latin; 'and others')</td>
</tr>
<tr>
<td>GRE</td>
<td>glycopeptide resistant enterococci</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HS</td>
<td>haemorrhagic septicaemia</td>
</tr>
<tr>
<td>IBR</td>
<td>infectious bovine rhinotracheitis virus</td>
</tr>
<tr>
<td>BCCM</td>
<td>Belgian Coordinated Collection of Microorganisms culture collection</td>
</tr>
<tr>
<td>M.</td>
<td>Mannheimia</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC of 50% of the investigated strains</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>MIC of 90% of the investigated strains</td>
</tr>
<tr>
<td>MLEE</td>
<td>multi-locus enzyme electrophoresis</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NCCLS</td>
<td>National Committee for Clinical Laboratory Standards (USA)</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures (UK)</td>
</tr>
<tr>
<td>NPG</td>
<td>β-glucosidase</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>ONPF</td>
<td>α-fucosidase</td>
</tr>
<tr>
<td>ONPG</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>OTC</td>
<td>oxytetracycline</td>
</tr>
<tr>
<td>p</td>
<td>level of significance</td>
</tr>
<tr>
<td>P.</td>
<td>Pasteurella</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PK/PD</td>
<td>pharmacokinetic/pharmacodynamic</td>
</tr>
<tr>
<td>RAPD</td>
<td>randomly amplified polymorphic DNA</td>
</tr>
<tr>
<td>REA</td>
<td>restriction endonuclease analysis</td>
</tr>
<tr>
<td>REP-PCR</td>
<td>repetitive extragenic palindromic PCR</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>s.l.</td>
<td>sensu lato</td>
</tr>
<tr>
<td>s.s.</td>
<td>sensu stricto</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>subsp., ss.</td>
<td>subspecies</td>
</tr>
<tr>
<td>tDNA-PCR</td>
<td>tRNA-intergenic spacer PCR</td>
</tr>
<tr>
<td>tet</td>
<td>tetracycline resistance gene</td>
</tr>
<tr>
<td>Tet</td>
<td>tetracycline resistance protein</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>TSI</td>
<td>triple sugar iron</td>
</tr>
<tr>
<td>UPGMA</td>
<td>unweighted pair group method with arithmetic mean</td>
</tr>
</tbody>
</table>
PREFACE

When applications derived from a human discovery are abundantly used, consequent implications are not always foreseen. A marked example of this, in both human and veterinary medicine, is the emergence of antimicrobial resistance following the use of antimicrobial drugs. These once reported ‘miracle drugs’ are nowadays jeopardized by resistance genes in the target bacteria, leading to therapy failure of important bacterial diseases. A potential relation of unknown magnitude between antimicrobial resistance in human and veterinary medicine further makes the topic a worldwide worrisome debate.

Many underlying resistance mechanisms, as well as the pathways in which resistance determinants can spread, are well documented in literature. However, the epidemiology of antimicrobial resistance that explains prevalence and describes contributing field factors in the development and maintenance of antimicrobial resistance is not yet fully understood. To explore this epidemiology with particular reference to livestock, detailed investigations at herd level are attributed to be a prerequisite.

In the here presented studies, attempts were made to identify contributing factors related to the problem of antimicrobial resistance with particular reference to bovine pasteurellosis. Pasteurella and Mannheimia organisms are the main causative organisms of this economically important disease, and are frequently reported to be resistant for the available antimicrobial agents required for a successful therapy. Veterinary clinicians have to deal with bovine pasteurellosis in particular herds, but the resistance situation of these important pathogens at herd level is far from fully revealed. When the occurrence and spread of resistance in Pasteurella and Mannheimia organisms is better documented at herd level, this information will help bovine practitioners to minimize the number of therapy failures of bovine pasteurellosis related to antimicrobial resistance.
CHAPTER 1.

GENERAL INTRODUCTION
Chapter 1.1.
1.1. REVIEW OF LITERATURE

One of the economically most important diseases in bovine livestock is pasteurellosis, a multifactorial respiratory disorder that mainly affects calves. As the name indicates, in many cases bacteria belonging to the family Pasteurellaceae are involved. These bacteria are part of the normal microbiota in the upper respiratory tract (Kehrenberg et al., 2001), making the disease difficult to prevent. For a veterinary practitioner, knowledge of the pathogen involved and its antimicrobial resistance profile is essential information for an adequate treatment. However, a substantial reclassification of bovine relevant Pasteurellaceae has been carried out since 1999, starting with the introduction of the new genus Mannheimia (Angen et al., 1999). Consequently, the accuracy of a routine bacterial diagnosis of bovine pasteurellosis, and of the epidemiology and the antimicrobial susceptibilities of Pasteurellaceae based on former investigations is questionable. Thus, there is a need to reinvestigate bovine Pasteurellaceae according to this reclassification assisted by currently indispensable molecular identification tools.

During the last two decades, several reports have documented antimicrobial resistant strains within bovine Pasteurellaceae isolated from clinical cases. Underlying resistance genes, resistance mechanisms, and possible pathways of spread have been described as well in these important bovine respiratory pathogens (Kehrenberg et al., 2005). However, many resistance genes and factors contributing to the emergence of antimicrobial resistance still remain to be discovered. This prevents the development of specific guidelines to limit the emergence of resistant Pasteurellaceae, and by consequence to minimize therapy failures due to bovine pasteurellosis.

1.1.1. CLASSIFICATION AND CHARACTERISTICS OF PASTEURELLACEAE ASSOCIATED WITH BOVINE DISEASE

Organisms belonging to the bacterial family Pasteurellaceae are ubiquitously present in the respiratory, alimentary and reproductive tracts of different avian, mammalian, reptilian, and likely amphibian hosts (Shewen and Rice Conlon, 1993; Rycroft and Garside, 2000; Christensen et al., 2003b; 2004b). Members of this family are small (0.2-2μm), Gram-negative, non-motile, facultatively anaerobic coccobacilli or rods. In addition they are, with few exceptions, fermentative and oxidase- and catalase-positive. Growth on artificial media is enhanced by the addition of serum or blood, on which they
appear after 24 hours of incubation as round, greyish colonies of moderate size (Shewen and Rice Conlon, 1993; Quinn et al., 1994). Ever since the isolation and recognition of the first pathogenic Pasteurella organism by Louis Pasteur at the end of the 19th century, a proper identification of members of the family Pasteurellaceae has been the source of much debate. Remarkably, and in contrast with other bacterial families of pathogenic importance, commercial identification kits were found not to be reliable for species identification of members of this bacterial family (Fajfar-Whetstone et al., 1995; Christensen et al., 2003b). During the last two decades, the combination of 16S rRNA sequencing and other molecular techniques (Dewhirst et al., 1992; Christensen and Bisgaard, 2004), has led to several new genera and over 58 formally recognized species and 25 unnamed taxa within the family Pasteurellaceae (Christensen and Bisgaard, 2004). The so far designated genera within this family are Pasteurella (Mutters et al., 1985a), Mannheimia (Angen et al., 1999), Actinobacillus (Christensen and Bisgaard, 2004), Haemophilus (Broom and Sneath, 1981), Histophilus (Angen et al., 2003), Lonepinella (Osawa et al., 1995), Phocoenobacter (Foster et al., 2000), Gallibacterium (Christensen et al., 2003a), Volucribacter (Christensen et al., 2004b), Nicoletella (Kuhnert et al., 2004) and Avibacterium (Blackall et al., 2005).

The most substantial reclassification in relation to bovine diseases was the redesignation of organisms formerly known as [Pasteurella] haemolytica into the new genus Mannheimia, with Mannheimia (M.) haemolytica as the type species besides four other new species (Table I). Organisms previously identified as [Pasteurella] haemolytica, have earlier been categorized into 3 biotypes (sugar fermentation pattern of trehalose (T) and arabinose (A)), 12 biogroups (extended phenotyping including fermentation patterns of sugars and glucosides), and 17 serotypes (surface antigens) (Shewen and Rice Conlon, 1993; Highlander, 2001; Angen et al., 2002). In 1985, [Pasteurella] haemolytica was excluded from the genus Pasteurella sensu stricto (s.s.) by means of DNA-DNA hybridization (Mutters et al., 1985a). Despite this, strains formerly designated as [Pasteurella] haemolytica biotype T (fermenting trehalose) were redefined in 1990 -and are currently still referred to- as [Pasteurella] trehalosi (Sneath and Stevens, 1990). A decennium later, a new genus Mannheimia including at least 5 species (Table I) was proposed for the remaining trehalose-negative taxa within the [Pasteurella] haemolytica complex (Angen et al., 1999). Older classifications are therefore no longer appropriate for species-specific diagnosis. Remarkably confusing, two of the most detailed studied serotypes of M. haemolytica sensu latu (s.l.), i.e. A1 and A6, do not ferment arabinose
(Angen et al., 1999) although the original affiliation (“A”) was based on this characteristic and this typing is currently still used in vaccination leaflets. Species-specific allocation of *M. haemolytica* (Angen et al., 1999) requires additional tests compared to the identification of *[Pasteurella] haemolytica* (Mutters et al., 1985a; Quinn et al., 1994), but routine laboratory diagnosis and many peer reviewed manuscripts still regard these two as synonyms. The clinical relevance of this inaccurate reporting is not known. In the remaining sections of this introductory chapter, the term “*Mannheimia haemolytica* s.l.” will be used when referring to investigations before the introduction of the genus *Mannheimia* (unless otherwise specified, e.g. *M. haemolytica* s.s., serotype A1), or where *M. haemolytica* was used as synonym for *[Pasteurella] haemolytica*.

Although for several groups of *Pasteurellaceae*, host range and disease manifestations are not constant (Bisgaard, 1993), the host animal species is indicative for routine bacterial identification (Christensen and Bisgaard, 2004). A list of species within the family *Pasteurellaceae* that are associated with ruminants, with emphasis on bovine diseases, is given in Table I. In accordance with other members of the *Pasteurellaceae* (Kuhnert et al., 2004; Christensen et al., 2004b), the predominant associated infections are localized in the respiratory tract or are to a lesser extent septicaemic in nature. The two most important and best studied members involved in bovine diseases are *M. haemolytica* s.l., and *Pasteurella (P.) multocida* (Watts et al., 1994; Kehrenberg et al., 2001, Duarte and Hamdan, 2004). In spite of the molecular advances, morphology and restricted phenotyping are routinely used for primary identification of *Pasteurellaceae*. Mostly, the primary identification of *M. haemolytica* s.l. is based on the isolation of round greyish colonies of moderate size with a small pronounced surrounding zone of haemolysis after 24h of aerobic incubation on sheep blood agar. *M. haemolytica* s.l. does not produce indole and tolerates bile salts. *P. multocida* typically grows as mucoid confluent colonies, and in routine diagnostic bacteriology it is distinguished from *M. haemolytica* s.l. by production of indole from tryptophan, and by lack of haemolysis. A typical sweetish odour can be present (Shewen and Rice Conlon, 1993; Quinn et al., 1994).

Whereas the habitat and the clinical relevance of *M. haemolytica* s.l. is predominantly restricted to ruminants, and more particularly cattle, *P. multocida* is also associated with diseases in several other animals, including wound infections and septicaemia in man (Hunt et al., 2000). Similarly to *M. haemolytica* s.l., attempts have been made to associate clinical manifestations and host specificity with certain strain types.
of *P. multocida*. Firstly, Mutters *et al.* (1985a) demonstrated that *P. multocida* can be categorized upon sugar fermentation patterns ( dulcitol and sorbitol) into three subspecies (subsp.) *multocida*, *septica*, and *gallicida*. However, host specificity can only be attributed to subsp. *gallicida*, which is a typical fowl-associated organism. In contrast, subsp. *septica* isolates are mainly recovered from dogs, cats, birds and man, while subsp. *multocida* causes significant diseases in many domestic animal species (Mutters *et al.*, 1985a; Quinn *et al.*, 1994). Although in 2002 a fourth novel subspecies *tigris* was proposed by Capitini *et al.* (2002) on the basis of 16S rRNA sequencing, this subspecies associated with an infected tiger bite has so far not been referred to in later investigations. A more vulnerable classification system for host and disease specificity is based on antigenic differences of capsular polysaccharides distinguishing 5 capsular serogroups (Rimler and Rhoades, 1989), possibly extended by differences in antigenic-variety of lipopolysaccharides into 16 somatic serotypes (Heddleston *et al.*, 1972). In general, capsular type A is mainly associated with fowl cholera, recurrent purulent rhinitis in rabbits (‘snuffles’), and ruminant, porcine, canine, and feline respiratory disorders. Although type D is the predominant capsular type associated with atrophic rhinitis of pigs, this strain is occasionally isolated from pneumatic lungs in other animal species, including ruminants. Capsular types B and E are restricted to haemorrhagic septicaemia in bovine species from tropical regions, while *P. multocida* capsular type F is isolated mainly from diseased turkeys (Shewen and Rice Conlon, 1993; Quinn *et al.*, 1994). However, in many studies *P. multocida* isolates were not typed to subspecies, serogroup or capsular type levels, leaving some epidemiological aspects of this species unclear.
<table>
<thead>
<tr>
<th>Taxa</th>
<th>Origin/Pathology</th>
<th>Former classification</th>
<th>Ref.</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinobacillus lignieresii</em></td>
<td>Bovine pyogranulomatous lesions (Actinobacillosis)</td>
<td></td>
<td>[5,7]</td>
<td></td>
</tr>
<tr>
<td><em>Actinobacillus</em>&lt;sup&gt;a&lt;/sup&gt; <em>seminis</em></td>
<td></td>
<td></td>
<td>[5,19]</td>
<td>Brackets indicate the species was excluded from the genus <em>sensu stricto.</em></td>
</tr>
<tr>
<td><em>Actinobacillus</em>&lt;sup&gt;a&lt;/sup&gt; <em>succinogenes</em></td>
<td>Bovine rumen (lesions not reported)</td>
<td></td>
<td>[10,17]</td>
<td></td>
</tr>
<tr>
<td><em>Histophilus somni</em></td>
<td>Bovine meningo-encephalitis, bovine (BRD&lt;sup&gt;c&lt;/sup&gt;), ovine and caprine pneumonia, septicaemia, mastitis, arthritis, otitis</td>
<td><em>[Haemophilus] somnus, Haemophilus agni, Histophilus ovis</em></td>
<td>[3,8,17]</td>
<td></td>
</tr>
<tr>
<td><em>Pasteurella</em>&lt;sup&gt;b&lt;/sup&gt; <em>aerogenes</em></td>
<td></td>
<td></td>
<td>[3,8,17]</td>
<td></td>
</tr>
<tr>
<td><em>Pasteurella lymphangitidis</em></td>
<td></td>
<td></td>
<td>[6]</td>
<td></td>
</tr>
<tr>
<td><em>Pasteurella</em>&lt;sup&gt;c&lt;/sup&gt; <em>multocida</em></td>
<td>capsular type A, D, capvar type B, E</td>
<td>Indole negative variants of <em>P. multocida:</em></td>
<td>[8,9,13,14,15,18]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pasteurella</em>&lt;sup&gt;a&lt;/sup&gt; <em>avium</em> biovar 2, <em>Pasteurella canis</em> biovar 2&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pasteurella</em>&lt;sup&gt;c&lt;/sup&gt; <em>trehalosi</em></td>
<td></td>
<td></td>
<td>[1,2,15,19]</td>
<td></td>
</tr>
<tr>
<td><em>Mannheimia</em> <em>glucosida</em></td>
<td></td>
<td><em>[Pasteurella] haemolytica</em> serogroups 3,4,10,15 (biotype T)</td>
<td>[1,2,15,19]</td>
<td></td>
</tr>
<tr>
<td><em>Mannheimia</em> <em>granulomatis</em></td>
<td></td>
<td><em>[Pasteurella] haemolytica</em> serogroups 2, 11 (biogroup 3A-3H,9)</td>
<td>[1,2,16]</td>
<td></td>
</tr>
<tr>
<td><em>Mannheimia</em> <em>haemolytica</em></td>
<td></td>
<td><em>[Pasteurella] haemolytica</em> serogroups 1,2,5-9,12-14,16 (biogroup 1)</td>
<td>[1,2,8,11,15]</td>
<td></td>
</tr>
<tr>
<td><em>Mannheimia</em> <em>ruminalis</em></td>
<td></td>
<td><em>[Pasteurella] haemolytica</em> (biogroup 8D), <em>Actinobacillus lignieresii</em></td>
<td>[1,2,16]</td>
<td></td>
</tr>
<tr>
<td><em>Mannheimia</em> <em>succiniproducens</em></td>
<td></td>
<td></td>
<td>[12]</td>
<td></td>
</tr>
<tr>
<td><em>Mannheimia</em> <em>varigena</em></td>
<td></td>
<td><em>[Pasteurella] haemolytica</em> (biogroup 6)</td>
<td>[1,4]</td>
<td></td>
</tr>
<tr>
<td><em>Mannheimia</em> spp.</td>
<td></td>
<td><em>[Pasteurella] haemolytica</em> (biogroups 8A-C,9,12)</td>
<td>[1,2]</td>
<td></td>
</tr>
</tbody>
</table>

1.1.2. MOLECULAR IDENTIFICATION OF PASTEURELLACEAE

Because culture conditions can influence the expression of morphology, sugar fermentation patterns and serological properties, phenotyping is found not to be a stable and reliable method for strain identification and subtyping of Pasteurella spp. (Hunt et al., 2000) and Mannheimia spp. (Angen et al., 2002) in a clinical or epidemiological context. Molecular identification bypasses these disadvantages, and will further improve accurateness of characterization (in pure and/or mixed cultures), speed of detection, determination of taxonomic position, and understanding of intra-species genetic relationships (Hunt et al., 2000). In an epidemiological context, accurate subtyping is important to recognize particularly virulent strains (in case of outbreaks or monitoring and vaccination programmes) and their source of origin (e.g. cross-transmission). In relation to antimicrobial resistance, molecular typing methods are essential to distinguish between clonal and horizontal spread of resistance genes and to monitor the occurrence and relatedness of resistant strains on an international scale (George, 1998; Aarts et al., 2001).

The ideal subtyping methodology must meet several criteria (Olive and Bean, 1999; Schwarz et al., 2003). Firstly, the tool should be able to type all organisms within a certain species (typability), and thus must not rely on the detection of a marker that is not always present (e.g. haemolysis after subculturing in Mannheimia, Barbour et al., 1997). Secondly, it needs a high discriminatory power and reproducibility, i.e. capable to demonstrate both relatedness and unrelatedness of strains, and able to yield the same result after repeated testing of the same strain, respectively. Thirdly, the system should be cost-effective and at least in part be automatizable, and the resulting data must be easily interpretable. Finally, it must be possible to exchange data between different laboratories for comparative purposes.

As mentioned in the previous section, the nomenclature or taxonomical position of most Pasteurellaceae relies on 16S rRNA sequencing. Because this methodology requires extended laboratory equipment and experience, the currently most applied alternative approaches are species-specific PCRs and molecular fingerprinting (Olive and Bean, 1999; Aarts et al., 2001). The first type of approach consists of species PCRs that amplify unique DNA sequences. They have been successfully developed for the toxA gene (Nagai et al., 1994), psl gene (Kasten et al., 1997), and KMT1 region (Townsend et al., 1998) in P. multocida, and are used predominantly in clinical specimens for diagnostic purposes.
Recently, Townsend et al. (2001) combined several of such regions (cap loci) to develop a multiplex capsular PCR typing system which is able to discriminate the five capsular types of *P. multocida*. The second approach, molecular fingerprinting, provides a unique 'signature' of a bacterial strain and has been used for the identification of microorganisms in a broad microbiological context. Fingerprinting techniques are briefly reviewed in Table II. Although many of these techniques were applied for *Pasteurellaceae*, the new genus *Mannheimia* was never included.
Table II. Comparison of molecular fingerprinting techniques applied for *Pasteurellaceae*.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PFGE</th>
<th>locus-specific RFLP</th>
<th>RAPD (AP)-PCR</th>
<th>REP-PCR</th>
<th>AFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typability</td>
<td>++</td>
<td>++(+)</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Discrimination power</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++(+)</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Interlaboratory exchangeability</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Time required (days)</td>
<td>3d</td>
<td>1d</td>
<td>1d</td>
<td>1d</td>
<td>2d</td>
</tr>
<tr>
<td>Ergonomics (&amp; cost$^c$)</td>
<td>+ (26.4 €)</td>
<td>++ (16.8 €)</td>
<td>+++ (13.2 €)</td>
<td>+++ (13.2 €)</td>
<td>++ (24.0 €)</td>
</tr>
<tr>
<td>Ref$^d$ for <em>Pasteurellaceae</em></td>
<td>[3,5]</td>
<td>[7,8]</td>
<td>[1,4]</td>
<td>[2,3,6,9]</td>
<td>[2,4]</td>
</tr>
</tbody>
</table>

1.1.3. DEFINITION AND DIAGNOSIS OF BOVINE PASTEURELLOSIS

Bovine pasteurellosis can be categorized into two well recognized disease manifestations according to their distinct geography and etiology. In tropical regions (Asia, Africa) the most encountered form is haemorrhagic septicaemia (HS), caused by *P. multocida* capsular types B and E (Shewen and Rice Conlon, 1993; Townsend et al., 1998). *P. multocida* capsular types B and E are attributed to be primary invaders in HS. In Western countries, the syndrome mostly manifests itself as a respiratory disease syndrome (pneumonic pasteurellosis) and *M. haemolytica* s.l. and *P. multocida* act herein as secondary invaders in processes initially triggered by viruses and predisposing circumstances (Kehrenberg et al., 2001; Catry et al., 2002). In the present section, we focus on the latter disease syndrome.

Bovine pneumonic pasteurellosis, also known as bovine respiratory disease (BRD), bovine enzootic bronchopneumonia (BEB), or respiratory disease complex of cattle, is a multifactorial disorder that mainly affects young animals. The diagnosis of bovine pasteurellosis relies firstly on the following typical clinical symptoms: nasal discharge, cough, dyspnoea, anorexia, and elevated temperature (Wikse and Baker, 1996). Mostly several animals (calves) within a certain herd are affected. BRD accounts for one of the economically most important diseases in bovine livestock, with a global mortality and morbidity that are estimated to exceed 1% and 10% of young cattle, respectively (Martin et al., 1981).

The multifactorial character of bovine pasteurellosis relates to predisposing factors, primary or intercurrent viral infections and the different bacterial agents involved. Predisposing factors are severe climate change and stress due to overcrowding and transport which are typically found in feedlots and veal calf industry (Wikse and Baker, 1996; Highlander, 2001). Following transport, the disease is also known as ‘shipping fever’. Generally speaking, both predisposing factors and viruses are capable of inducing impaired pulmonary defences, which allows secondary infections mostly by opportunistic pathogenic *Pasteurellaceae* (Wikse and Baker, 1996; Catry et al., 2002).

Worldwide, two organisms belonging to the bacterial family *Pasteurellaceae* are recognized to be of major importance in bovine pasteurellosis, *M. haemolytica* s.l., and *P. multocida*. Contrary to the situation in Belgium (Catry et al., 2002), a third member of the *Pasteurellaceae*, namely *Histophilus somni* is also frequently involved in countries such as...
Canada and the U.S.A. (Allen et al., 1992; Welsh et al., 2004). All these bacteria are called opportunistic or facultative pathogenic because calves harbour them in the upper respiratory tract (Allen et al., 1991; Shwen and Rice Conlon, 1993; Quinn et al., 1994). The best studied organism is \[ Pasteurella \] haemolytica serotype “A”1, because it is recognized to be the most virulent pathogen (Highlander, 2001). Because of the recent reclassification of \( M. \) haemolytica s.l. into at least five new species (Angen et al., 1999), it is currently unclear which of the latter species calves harbour in the upper respiratory tract. \( P. \) multocida and \( H. \) somni are thought to be individually less invasive and associated bronchopneumonias are attributed to be less acute and fulminating (Shwen and Rice Conlon, 1993; Wikse and Baker, 1996; Tegtmeier et al., 2000; Welsh et al., 2004).

Two older studies (Allan et al., 1985; Allen et al., 1991) investigated whether nasal cultures can be predictive for the pathogen and its antimicrobial susceptibility profile present in the lungs of affected calves, because the airborne transmission during infection would suggest that the pathogenic organism in the lungs would be clonal in nature to the organism in the nasopharynx. Although a certain agreement was present at pen level, the authors failed to find a good correlation between nose and lung cultures at the individual animal level (Allan et al., 1985; Allen et al., 1991). More recently, DeRosa et al. (2000) demonstrated by means of molecular techniques a genetically identical organism in approximately 70% of 40 paired samples. In addition, the antimicrobial susceptibilities of these paired isolates showed an even higher degree of similarity. The authors concluded that nasal cultures of acutely sick animals are reliable to guide practitioners in the treatment of pneumonic pasteurellosis, especially with regard to antibiotic susceptibility.

Prevention of the disease is based on minimizing the predisposing factors and vaccination. Vaccines have been worldwide developed for \( M. \) haemolytica s.l., \( P. \) multocida and \( H. \) somni (Welsh et al., 2004). Vaccination can reduce disease and improve production, but it should be stressed that protection against BRD is far from absolute. This is likely due to the complex multifactorial origin of the syndrome that include environmental factors like population density, housing conditions, climate changes, stress, and a wide variety of etiological microorganisms like viruses, \( Pasteurellaceae \), and \( Mycoplasma \) spp. Vaccines are mostly focused on one or only a small number of serotypes and cross-protection does not always occur (Coomber et al., 2001; Highlander, 2001). In contrast, vaccination against haemorrhagic septicaemia is a very effective prevention as a result of the capsular serotype-specificity of the \( P. \) multocida organisms associated with
Review

this manifestation of bovine pasteurellosis (Shewen and Rice Conlon, 1993). In Belgium, three vaccines for the prevention of bovine pneumatic pasteurellosis are available, although none of these offer protection against *P. multocida* or *H. somni*. The currently registered Belgian products include vaccines against *M. haemolytica* serotype “A1” alone, or in combination with serotype “A6” or with BRSV (bovine respiratory syncytial virus) and BPI3 (bovine parainfluenza virus type 3). Despite the availability of vaccines against all three major bovine respiratory pathogens in the U.S.A., this practice remains far from routinely performed (Welsh *et al.*, 2004). As a consequence of this, and the ineffectiveness of vaccines because of the multifactorial nature of BRD, the currently most effective control method for bovine pasteurellosis is antimicrobial therapy. Antimicrobial agents can be administered either alone or in combination with anti-inflammatory drugs, mucolytica, or pulmonary function sustaining drugs (e.g. atropine, diuretica) (Wikse and Baker, 1996).

1.1.4. **ANTIMICROBIAL THERAPY AND RESISTANCE MONITORING FOR BOVINE PASTEURELLOSIS**

To combat bovine pneumatic pasteurellosis, application of antibiotics may be performed for either therapeutic, prophylactic, or metaphylactic purposes (Schwarz and Chaslus-Dancla, 2001). Whereas curative therapy indicates the treatment of the individual animal in which the diagnosis is made, prophylactic administration is frequently performed in the U.S.A. in the prevention of shipping fever prior to transport into feedlots. In Europe, prophylactic administration is element of medicated starter rations on veal calf farms after arrival from the native farm. Metaphylaxis is the term used for the treatment of subclinically affected animals in the direct surroundings of diseased animals in order to reduce the morbidity and the mortality of an outbreak. Also the latter 'convenience' method is typical for intensive rearing systems like feedlots and veal calf industry and in many cases metaphylactic treatment is administered orally. As outlined in depth in a following section (epidemiology of antimicrobial resistance), it has been demonstrated that especially orally administered antimicrobials during long periods will result in resistant commensal bacteria in the intestinal tract (Catry *et al.* 2003).

The variety of compounds nowadays available to control bovine pasteurellosis is substantial. Examples are beta-lactam antibiotics such as aminopenicillins (+clavulanic acid) and extended spectrum cephalosporins (cefquinome, ceftiofur), tetracyclines
(oxytetracycline, doxycycline), potentiated sulfonamides (trimethoprim + sulfonamides), macrolides (tylosin, tilmicosin, tulathromycin), aminoglycosides (gentamicin), aminocyclitols (spectinomycin), lincosamides (lincomycin), phenicols (florfenicol), and second generation fluoroquinolones (marbofloxacin, danofloxacin, enrofloxacin). However, decreased susceptibility and antimicrobial resistance is frequently reported for many of these agents in Pasteurella and Mannheimia organisms isolated from pneumonic lungs of calves (Watts et al., 1994; Kehrenberg et al., 2001).

The ultimate goal of antimicrobial therapy in the context of bovine pasteurellosis is to achieve a concentration in the lungs (or blood in case of haemorrhagic septicaemia) that has an inhibitory effect on target bacteria such as M. haemolytica s.l. and P. multocida. Then clinical recovery of the affected animal is enhanced. The first indication to assess whether a certain bacterium will be inhibited by a certain antimicrobial agent is testing its susceptibility through determination of its minimum inhibitory concentration (MIC) for that compound. The MIC is defined as the lowest concentration of an antimicrobial agent that visibly inhibits growth under standardized laboratory conditions. If the isolate shows a MIC that is equal to or less than an approved clinical breakpoint for susceptibility, the isolate is considered as susceptible and an appropriate antimicrobial therapy is likely to be effective. When higher MIC values are measured, the corresponding isolates will be classified as either intermediate susceptible or as resistant. In both cases, the administration of the respective antimicrobial agent to an animal infected by such a resistant organism is unlikely to be efficacious. For P. multocida and M. haemolytica s.l. isolates associated with BRD, the antimicrobials for which internationally approved veterinary-specific breakpoints exist that allow the classification of the organisms as “susceptible”, “intermediate” and “resistant” include florfenicol, enrofloxacin, ceftiofur, spectinomycin and tilmicosin (NCCLS, 2002, 2004). In other words, for the majority of antimicrobial agents widely used for the treatment of BRD, there is paradoxically no approved system to differentiate between susceptible and resistant P. multocida and M. haemolytica s.l. isolates. The establishment of an internationally approved breakpoint, and in general the attribution to designate a bacterium as susceptible or resistant for a certain antimicrobial drug, depends on three main criteria. A microbiological criterium (1) based on MIC determinations of large sets of the organism of interest, a pharmacological criterium (2) derived from plasma and tissue (lung) concentrations of a certain compound (pharmacokinetics), and a clinical criterium (3) that relies on efficacy of the molecule under experimental or field conditions (field trials). Once an organism is designated to be primarily (intrinsically) susceptible to a
certain compound, an alteration in the bacterial genome can however result in acquired resistance (Catry et al., 2003). The demonstration of this alteration in the bacterial genome, and the verification that it is responsible for the decreased susceptibility or resistance is a currently recognized fourth molecular criterion. If no approved breakpoints for a certain bacterium-antimicrobial combination are available, the latter criterion can be helpful to discriminate between resistance and susceptibility.

To keep veterinary practitioners informed on the abundance of resistant *Pasteurella* and *Mannheimia* strains indicative for therapy failure, regularly resistance monitoring of clinical cases of BRD is required on a regional basis. During such a monitoring study in Belgium in which 80 affected calves prior to treatment were examined (Catry et al., 2002), a remarkable low incidence of resistance was found compared to the surrounding countries (Table III). The Belgian study concluded with the hypothesis that these differences resulted from the fact that in neighbouring countries necropsy strains prevailed in the collections studied, since they harbour more frequently resistance genes as a consequence of the antimicrobial resistance selection pressure during the course of the disease. From an epidemiological and a clinical point of view, the intriguing question arose whether resistant subpopulations of *Mannheimia* or *Pasteurella* organisms are present prior to an outbreak of BRD, but remained undetected during the Belgium study as a consequence of the methodology used.

Table III illustrates that tetracycline resistance is among the most frequently reported resistance properties among *Pasteurella* and *Mannheimia* organisms. This class of antimicrobial agents inhibits bacterial growth by reversely binding to the bacterial ribosome. It is worldwide one of the most commonly used antimicrobial agents (Schwarz and Chaslus-Dancla, 2001). Consequently, detection systems for the underlying resistance genes (Table IV) and their localization in the chromosome are well established for Pasteurellaceae (Hansen et al., 1996; Kehrenberg et al., 2001; Kehrenberg et al., 2005). It is therefore an interesting molecule to focus on, when investigating antimicrobial resistance related to bovine pasteurellosis.
**Table III.** Susceptibility percentages of clinical bovine *P. multocida* en *M. haemolytica* s.l. isolates (Catry *et al.*, 2002).

<table>
<thead>
<tr>
<th>Country</th>
<th>Period</th>
<th>AMP(^a)</th>
<th>CEF</th>
<th>TET</th>
<th>TMP/S</th>
<th>FLOR</th>
<th>ENRO</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. multocida</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Belgium</td>
<td>1999-2002</td>
<td>100 (43)(^b)</td>
<td>100 (43)</td>
<td>97.7 (43)</td>
<td>95.4 (43)</td>
<td>100 (43)</td>
<td>100 (43)</td>
</tr>
<tr>
<td>Netherlands</td>
<td>1996-1997</td>
<td>91 (58)</td>
<td>100 (57)</td>
<td>55 (60)</td>
<td>55 (58)</td>
<td>100 (83)</td>
<td>95 (60)</td>
</tr>
<tr>
<td>France</td>
<td>1995-1997</td>
<td>90 (678)</td>
<td>-</td>
<td>64 (804)</td>
<td>85 (617)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Germany</td>
<td>2000-2001</td>
<td>89.6 (106)</td>
<td>100 (45)</td>
<td>44.3 (106)</td>
<td>91.5 (106)</td>
<td>98.1 (106)</td>
<td>100 (106)</td>
</tr>
</tbody>
</table>

| *M. haemolytica* |              |            |     |      |       |       |       |
| Belgium | 1999-2002    | 100 (11)  | 100 (11) | 100 (11) | 100 (11) | -     | 100 (11) |
| Netherlands | 1996-1997   | 58 (74)   | 100 (74) | 47 (75)  | 42 (74)  | 100 (60) | 95 (74)  |
| France  | 1995-1997    | 40 (1000) | -   | 39 (1015) | 68 (834) | -     | -     |
| Germany | 2000-2001    | 88.1 (59) | 100 (37) | 20.3 (59) | 59.3 (59) | 96.6 (59) | 100 (59) |

\(^a\)AMP: ampicillin, CEF: ceftriaxone, TET: oxytetracycline, TMP/S: trimethoprim + sulfonamides, FLOR: florfenicol, ENRO: enrofloxacin. \(^b\)Susceptibility percentages, between brackets: number of isolates.
1.1.5. **FUNDAMENTALS OF ANTIMICROBIAL RESISTANCE IN RELATION TO BOVINE PASTEURELLOSIS**

1.1.5.1. **MOLECULAR BASIS, MECHANISMS, AND SPREAD OF ANTIMICROBIAL RESISTANCE**

Acquired resistance to an antimicrobial drug is the result of an alteration in the genome of a microorganism. The primary pathway, through which the alteration of the genome can occur, is the mutation of a gene into a resistance gene or region (*de novo* resistance). This is usually a long-term process that involves a number of mutational steps, although single-step mutations may also account for resistance to certain antimicrobial agents (Schwarz and Chaslus-Dancla, 2001). The stepwise resistance development is best demonstrated for genes coding for inactivating enzymes (e.g. phosphotransferases, acetyltransferases) that are likely to be derived from house-keeping genes of the bacterial cell. It is also well documented for resistance to quinolones within a wide variety of organisms (Catry *et al.*, 2003). Mutations or changes in the genome, mostly in the chromosome, occur spontaneously during the replication of the bacterial genome. The secondary pathway is the transfer of resistance genes from donor bacteria into acceptor bacteria via conjugation/mobilisation, transformation or transduction. These three processes constitute the three different forms of horizontal gene transfer. Conjugation/mobilisation is the most important transfer mechanism (Hawkey, 1998; Rowe-Magnus and Mazel, 1999). The rapid horizontal gene transfer which occurs in conjugation consists of the spread of mobile genetic elements such as plasmids (able to replicate autonomously), transposons (not able to replicate autonomously, movement by transposases) or gene cassettes (not able to replicate autonomously, movement by site-specific recombination) (Hall and Collins, 1995; Bennett, 1999), on any of which several resistance genes can be located (Catry *et al.*, 2003). Regarding the efficiency in the spread of antimicrobial resistance, the localization of these resistance determinants in the genome is important. In general, chromosomal resistance genes spread vertically (clonal spread), while resistance genes on transposons, gene cassettes or plasmids can be disseminated horizontally as well as vertically. Therefore the latter spreading mechanism is more efficient (Kruse, 1999; Berends *et al.*, 2001). In favour of the spread of their resistance genes, movable elements like transposons can also be located on the chromosome.

The transferable resistance determinants originate from mutated bacterial housekeeping genes (essential for metabolic and fertility functions) and, more importantly,
from natural resistance genes originating from antibiotic producing microorganisms (e.g. *Streptomyces* spp.) (Beneviste and Davies, 1973; Shaw *et al*., 1993; Davies, 1994; Marshall *et al*., 1997). Horizontal gene transfer has contributed to the wide dissemination of these determinants in evolutionarily and ecologically distinctive bacteria (Kruse, 1999). For instance, this transfer has been demonstrated *in vitro* between gram-negative and gram-positive bacteria (Courvalin, 1994), and *in vivo* between the bacterial flora of animals and humans (Kruse and Sørum, 1994; van den Bogaard and Stobberingh, 1999).

Different resistance mechanisms are related to the method of action of different antimicrobial drugs (Table IV). The most important resistance mechanisms are antimicrobial drug modification, reduced intracellular accumulation, and modification of the target site (Catry *et al*., 2003). Antimicrobial drug modification consists of an enzymatic modification or inactivation of the antimicrobial drug that prevents the molecule from reaching the target site. The inactivation of the aminoglycoside streptomycin and the aminocyclitol spectinomycin by an adenyltransferase encoded by an *aadA1* gene, as identified in an avian *P. multocida* strain (Wu *et al*., 2003), is an example of enzymatic inactivation. The only example of tetracycline resistance due to enzymatic inactivation is coded by the *tet*(X) gene found in *Bacteroides* spp. on a transposon (Roberts, 1996). The second mechanism arises from an alteration in bacterial cell wall porins, resulting in either a decreased uptake or an increased removal of the antimicrobial drug from the cell. The reduced intracellular accumulation inhibits the antimicrobial drug from proceeding with its normal intracellular action. An increased removal of tetracyclines, for example, can be caused by the presence of the tetracycline efflux gene *tet*(B), which was found in porcine *P. aerogenes* isolates (Kehrenberg and Schwarz, 2001). The third resistance mechanism results from a modification of the target site. For instance, *tet*(M) found in bovine *P. multocida* isolates (Chaslus-Dancla *et al*., 1995; Hanssen *et al*., 1996), codes for proteins that inhibit tetracyclines to bind at the ribosome (Roberts, 1996). Most *tet* genes induce resistance for structurally closely related compounds e.g. oxytetracycline, doxycycline, and chlortetracycline. Cross-resistance can however also occur between structurally-unrelated molecules, when these compounds share the same target site, and this target site is modified by the product of a particular resistance gene (Schwarz *et al*., 2001). For instance, *erm* genes encode resistance to macrolides, lincosamides and B compounds of streptogramins (Martel *et al*., 2001; De Leener *et al*., 2004). Such solitary genes causing cross-resistance, or different resistance genes gathered on a single mobile genetic element
(transposons or plasmids), may cause co-selection (Catry et al., 2003). With co-selection, resistance to several different antimicrobial drugs can emerge very efficiently while only one antimicrobial drug is used.

An acquired resistance gene induces a resistance mechanism if this gene (or region) is expressed. For efflux-mediated tetracycline resistance for instance, a specific gene \( \textit{tet} \) codes for membrane-associated proteins (Tet) which exchange a proton for a tetracycline-cation complex (Roberts, 1996). Besides, resistance mechanisms can be either constitutive or inducible, depending on whether the resistance gene is always expressed or can be expressed in the presence of the antimicrobial drug. Most resistance mechanisms are constitutive. Inducible resistance is well documented for macrolides, and for two groups of tetracycline efflux genes (Roberts, 1996; Butaye et al., 2003). The first of the latter groups is present in \textit{Pasteurellaceae} that harbour the \( \text{tet(B)} \), \( \text{tet(G)} \), or \( \text{tet(H)} \) genes (Table IV). These structural \( \text{tet} \) genes are induced when tetracyclines, even in nanomolar amounts, inhibit the function of a protein (TetR) encoded by an accompanying repressor gene, called \( \text{tetR} \). In the absence of tetracyclines, the \( \text{tetR} \) gene blocks the transcription of the structural \( \text{tet} \) gene. The second group of inducible tetracycline efflux genes comprises \( \text{tet(L)} \) and \( \text{tet(K)} \). Both are mainly found in Gram-positive bacteria, and no repressor gene is involved in the induction (Butaye \textit{et al.}, 2003). Here, the induction is regulated by attenuation of the mRNA, also referred to as translation attenuation, a posttranscriptional regulatory device (Lovett, 1990).
Table IV. Resistance genes/regions and mechanisms found in *Pasteurella* and *Mannheimia* spp. (Kehrenberg *et al.*, 2005).

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Resistance mechanism</th>
<th>Resistance gene/region</th>
<th>Bacterial source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Pasteurella</em></td>
</tr>
<tr>
<td>Penicillins</td>
<td>β-lactamase</td>
<td><em>bla</em>&lt;sub&gt;ROB-1&lt;/sub&gt;</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>bla</em>&lt;sub&gt;TEM-1&lt;/sub&gt;</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>bla</em>&lt;sub&gt;PE1&lt;/sub&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>dihydropteroate synthase</td>
<td><em>sul2</em></td>
<td>+</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>dihydrofolate reductase</td>
<td><em>dfra20</em></td>
<td>+</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>efflux protein</td>
<td><em>tet</em>(B)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>tet</em>(H)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>tet</em>(G)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ribosome protective protein</td>
<td><em>tet</em>(M)</td>
<td>+</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>phosphotransferase</td>
<td><em>strA</em></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>strB</em></td>
<td>+</td>
</tr>
<tr>
<td>Streptomycin/spectinomycin</td>
<td>adenyltransferase</td>
<td><em>aadA1</em></td>
<td>+</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>acetyltransferase</td>
<td><em>catA1</em></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>catA3</em></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>catB2</em></td>
<td>+</td>
</tr>
<tr>
<td>Chloramphenicol/florfenicol</td>
<td>efflux protein</td>
<td><em>floR</em></td>
<td>+</td>
</tr>
<tr>
<td>Quinolones (naladixic acid)</td>
<td>mutation</td>
<td><em>gyrA</em></td>
<td>+</td>
</tr>
</tbody>
</table>
1.1.5.2. EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE

In a bacterial population, the three conditions responsible for the rapid spread of antimicrobial resistance are the presence of (or a mutation into) resistance genes, vertical (clonal) or horizontal spread of these resistance genes, and selection pressure (Schwarz et al., 2001). The selection pressure determines the rate and extent of the emergence of antimicrobial resistance, because it alters bacterial populations through the elimination of susceptible bacteria and the survival of resistant bacteria. The use of antimicrobials is the main cause of selection pressure (Damon, 1999; Wegener et al., 1999). Pathogenic organisms are clearly the target bacterial population of antimicrobial drugs, on which by consequence a selection pressure can be exerted. It is important to realize, however, that antimicrobial drugs also exert selection pressure on commensal bacteria (Barbosa and Levy, 2000) present in every individual on the skin, conjunctiva, in the upper respiratory tract, the lower urogenital tract, and especially in the digestive tract. Within the digestive tract, the largest reservoir of commensal bacteria is the intestinal tract (Berends et al., 2001; Sørum and Sunde, 2001). Consequently, excreted faecal bacteria -representing the intestinal tract microbiota- are investigated in the majority of studies on antimicrobial resistance. In addition, the measured levels of antimicrobial resistance in faecal commensal bacteria can reflect the selection pressure exerted by the use of antimicrobial agents in a certain environment (van den Bogaard and Stobberingh, 1999). In livestock, *Escherichia coli* and enterococci (*Enterococcus faecium* and *E. faecalis*) are therefore internationally used as Gram-negative and Gram-positive indicator bacteria, respectively, because of their high prevalence in the faeces of healthy animals and because of their ability to often harbour several resistance genes (Aarestrup, 2000; Wray and Gnanou, 2000; Catry et al., 2003). The purpose of monitoring antimicrobial resistance through indicator bacteria is to avoid misjudging (overestimating) resistance levels. Monitoring antimicrobial resistance in pathogenic bacteria may be less accurate because the resistance patterns of pathogenic strains isolated from necropsy or following therapy failure can be altered by the preceding antimicrobial treatment (Lester et al., 1990; van den Bogaard and Stobberingh, 1999). The contrast between pathogenicity and commensalism is flattened in relation to bovine *Pasteurellaceae* since they are part of the commensal respiratory flora and since they are opportunistic respiratory pathogens (Shewen and Rice Conlon, 1993), which make them a remarkable and intriguing bacterial population for research.
When focusing on the main trigger for the antimicrobial resistance selection, a first major antimicrobial drug-related factor is the treatment regimen. The regimen principally consists of the dose, the treatment interval, the duration of treatment, and the formulation, all of which influence the selection pressure on a bacterial population. Dose, as a risk factor, can in the first place be interpreted as the total amount of active substance used in a population during a certain time period (Lester et al. 1990; Austin et al., 1999; Wegener et al., 1999). Secondly, it can be interpreted as correctly dosed or not. In human medicine for instance, the long-term under-dosing of β-lactam antibiotics has clearly increased the risk of carriage of resistant bacteria in comparison with short-term high dosing of the drug (Guillemot et al., 1998). The aforementioned regimen is similar to the intensive use of antimicrobial growth promoters (AMGPs) in livestock animals. AMGPs have been used for decades to enhance growth, improve feed conversion and decrease waste production. In practice this consists of the prolonged oral administration of low dosages of antimicrobial drugs, especially in swine, poultry and veal calves (Bates et al., 1994; McEwen and Fedorka-Cray, 2002). Indeed, numerous studies have confirmed the presence of acquired resistance among commensal bacteria in the digestive tract of poultry and swine, and their association with the use of AMGPs (Levy et al., 1976; Gelling et al., 1989; Dunlop et al., 1998; Sunde et al., 1998; van den Bogaard et al., 2000; Swartz, 2002).

The optimal route of administration of an antimicrobial drug depends not only on the drug's formulation, but also on which route lends itself least to the emergence of antimicrobial resistance. In swine, Dunlop et al. (1998) found a significantly higher prevalence of resistant faecal coliforms in pigs receiving oral ampicillin and tetracyclines in comparison with individually and parenterally treated animals. The oral administration of a (broad spectrum) antimicrobial drug leads to the emergence of resistant bacteria (e.g. Enterobacteriaceae) in the gastro-intestinal tract within 7 days (Levy et al., 1976; Guggenbichler et al., 1985). A parenterally administered antimicrobial drug will exert a selection pressure in the infected body compartment, while the effect on the gastro-intestinal tract or other microbiota-containing surfaces depends on pharmacokinetic characteristics. In the gastro-intestinal tract, such effect relies on the extent to which the product is excreted as an active metabolite through the entero-hepatic cycle (Guggenbichler et al., 1985), a well documented phenomenon for tetracyclines.

Concerning the effect of the type of antimicrobial drug, antimicrobial agents have been categorized for a long time as either bacteriostatic or bactericidal, depending on their
predominant *in vitro* action. This distinction (taking clinically attainable concentrations into account) is not absolute since the *in vivo* action depends more on drug concentration attained in the target tissue and the pathogen involved (Dowling, 1996). In bovine bronchopneumonia, for instance, tetracyclines are bacteriostatic for *P. multocida*, but bactericidal for *M. haemolytica* (Norcia *et al.*, 1999). Nevertheless, in specific human conditions (endocarditis, meningitis, immunocompromised patients) the value of bactericidal (killing) agents is well established (Dagan *et al.*, 2001). Since bacteriostatic agents inhibit the growth of bacteria and do not kill them, clinical and bacteriological cure depends on the integrity of host defences (Lees and Shojape Aliabadi, 2000). In relation to antimicrobial resistance, one can assume that a cidal action, in contrast to a static action, is more likely to eradicate pathogens and by consequence more likely to reduce the risk of selection for antimicrobial resistance. In addition, failure to eliminate (eradicate) bacteria may promote the selection of antimicrobial resistant subpopulations (Dagan *et al.*, 2001). On the other hand, if a resistant subpopulation is present, killing susceptible bacteria (bactericidal action) while allowing resistant mutants to live could actually speed the selection process of antimicrobial resistance. Reconsidering bovine pneumonia, the overall prevalence of tetracycline-resistant *M. haemolytica* is indeed higher compared to tetracycline-resistant *P. multocida* (Table III).

To address the relationship between the type of drug and the antimicrobial resistance, attention has been given to the distinction between time-dependent (concentration-independent) and concentration-dependent killing agents. This distinction is based on their most important pharmacokinetic/pharmacodynamic (PK/PD) predictor (surrogate relationship) for clinical efficacy (Hyatt *et al.*, 1995; Lees and Shojape Aliabadi, 2000, Woodnut, 2000). Time-dependent killing is dependent on the time an antibiotic exceeds the minimum inhibitory concentration of the pathogen (PK/PD predictor = $T > \text{MIC}$), but is independent of the antibiotic concentration once the inhibitory level has been reached. For time-dependent antimicrobial agents ($\beta$-lactams, macrolides), increasing the period of time above the MIC at the site of infection for a minimum of 40-60 % of the dosing interval (depending on the molecule and the pathogen involved) is the key parameter for clinical efficacy (Craig, 1998) and should help to prevent the emergence of resistance (Hyatt *et al.*, 1995; Auckenthaler, 2002). However, if at a given infection site a considerable resistant subpopulation exists or arises (e.g. due to inducible $\beta$-lactamases in *Enterobacteriaceae*), it is plausible that, even if the concentration above the MIC is attained for a long period, neither bacteriological cure nor the avoidance of spread of these
resistant clones can be achieved through the use of time-dependent agents. The effectiveness of PD/PK parameters as predictors for the development of antimicrobial resistance is more clearly established in concentration-dependent agents (fluoroquinolones, aminoglycosides), with emphasis on fluoroquinolones, both in human (Moore et al., 1987; Forrest et al., 1993; Thomas et al., 1998) and animal studies (Drusano et al., 1993; Lees and Shojaee Aliabadi, 2002). In concentration-dependent agents, the primary determinant of clinical efficacy is the concentration of the drug at the site of infection, and not the frequency of dosing. Higher concentrations of a concentration-dependent antimicrobial drug kill the pathogen more quickly and more completely (eradication). Two PK/PD predictors have shown themselves to be important for clinical efficacy and to reduce the development of antimicrobial resistance. First, the ratio of the peak concentration over the MIC (C_max/MIC) that needs to be at least 8. The second predictor is the area under the inhibitory curve (AUIC), which must exceed 125, where AUIC is termed the 24-hour AUC divided by the MIC (AUC0-24/MIC) (Catry et al., 2003).

Within an animal species, age is a factor that influences the prevalence of antimicrobial resistance. Faecal coliforms in children (Lester et al., 1990; Calva et al., 1996; Kollef, 2000), in piglets (Langlois et al., 1988) and in calves (Wierup, 1975; Berge et al., 2001), as well as caecal enterococci in young chickens (Butaye et al., 1998) exhibit a higher prevalence of antimicrobial resistance than in full-grown man/animals. The reason for this age-related phenomenon is not yet understood.

In general, a different localization of a bacterial species within the host is correlated with a different prevalence of antimicrobial resistance. It has been found that the relative abundance of a resistant microorganism in a given organ system depends on the extent to which the system is able to harbour a large commensal flora (Martel and Vandaele, 1999; Dagan et al., 2001). For instance, pathogenic strains from the respiratory tract in cattle (Pasteurellaceae) have overall a higher degree of acquired resistance compared to pathogens from the udder (staphylococci and streptococci), although local antimicrobial therapy in the udder is a common practice. One plausible explanation for this fact is that the udder, in contrast to the respiratory tract (nasopharynx), does not have resident flora harbouring a reservoir of resistance genes (Martel and Vandaele, 1999).

Langlois et al. (1988) found that also housing conditions (production type) influence the prevalence of resistant faecal coliforms in swine. A higher level of resistant coliforms was found in finishing unit pigs than in pigs in the farrowing house or on...
pasture. Also in swine, Butaye et al. (1998) observed a difference in prevalence in glycopeptide resistant enterococci (GRE) between sows and fatteners. Similarly, faecal coliforms (Berge et al., 2001) and Pasteurellaceae from the respiratory tract (Mevius and Hartman, 2000) in fattening calves tend to show a higher degree of resistance than those in dairy calves. Contact intensity between a certain animal population in an ecological niche and a certain environment where antimicrobials are used (by man), seems thus to be associated with the level of antimicrobial resistance seen in that animal population (Catry et al., 2003). Walson et al. (2001) also observed a significantly decreased frequency of antimicrobial resistance with decreasing human population density.

1.1.5.3. IMPACT ON PUBLIC HEALTH

It is generally accepted that antimicrobial resistance in bacteria from animals could represent a potential public health hazard. In human medicine, antimicrobial resistance and especially the multiple resistances of Staphylococcus aureus, pneumococci, enterococci and Enterobacteriaceae isolated both from nosocomial and non-hospital-related infections (Swartz, 1994; Bronzwaer et al., 2001) have been found to cause therapy failure leading to higher morbidity and mortality rates (Holmberg et al., 1987; Acar, 1997; Kessler, 1997). Nowadays, opportunistic pathogens like Pseudomonas and Acinetobacter spp. worsen this situation because they are often multi-resistant and frequently isolated from infections in immunocompromised patients (Li et al., 2005). Due to demographic ageing, HIV, and the rising number of cancer treatments and transplantations, the number of immunocompromised patients is unlikely to decrease in the near future.

The public health aspect relates to the evidence that the commensal gastro-intestinal microbiota (represented by indicator bacteria) of healthy animals harbour a reservoir of resistance genes (Helmuth and Protz, 1997; van den Bogaard and Stobberingh, 1999). Animal associated resistant bacteria might temporarily colonize the human digestive tract through the food chain or by direct contact. There the underlying resistance genes can be transferred into bacteria belonging to human gastro-intestinal microbiota. If, moreover, this lateral transfer takes place into human pathogenic bacteria, this can result in treatment failure as a consequence of antimicrobial resistance (van den Bogaard and Stobberingh, 1999). The importance of this indirect path of resistance transfer is less clear than for the direct transfer of resistant zoonotic organisms. Because livestock animals can act as carriers of zoonotic pathogens such as Salmonella and Campylobacter spp., these
Chapter 1.1.

bacteria also undergo similar selection pressure due to the use of antimicrobial drugs. As a result, treatment failure in humans may arise as a consequence of the intake of these selected resistant organisms either through the food chain and/or through direct contact (Kruse, 1999), irrespective of the horizontal transfer of resistance genes.

Because of its association with the resistance observed in indicator bacteria isolated from livestock animals, the use of antimicrobial AMGPs (in particular) has become a source of much debate (Bates et al., 1994; van den Bogaard and Stobbering, 1999; Swartz, 2002). If the prevalence of resistance in the commensal faecal flora of livestock animals to a certain antimicrobial drug increases rapidly, the antimicrobial agent in question can be banned from use as an AMGP (Swartz, 2002). In accordance with the precautionary principle, up to now only four molecules remain accepted for use as AMGP in the European Union: monensin-Na, salinomycin-Na, flavophospholipol, and avilamycin. Only the latter two have true antibiotic activity (Butaye, 2000). These four antimicrobial agents are considered unlikely to exhibit cross-resistance with therapeutic compounds used in human and veterinary medicine (Schwarz et al., 2001). Thus the risk for co-selection of antimicrobial resistance for structurally related molecules is minimized. This precautionary measure, however, does not preclude the other pathway of co-selection through linked resistance genes. To further control the emergence of antimicrobial resistance, the four aforementioned compounds are planned to be withdrawn as AMGP from January 2006 in the European Union (Regulation (EC) No. 1831/2003).

One needs to be aware that selection is a natural process resulting from the use of antimicrobial drug consumption as well in human and veterinary medicine, as in aquaculture and horticulture (Catry et al., 2003). Horizontal transfer of resistance genes between commensal bacteria from livestock and human pathogens can speed up the emergence of resistance but is seldomly documented. Thus, the impact of the veterinary use of antimicrobial drugs on resistance problems in human medicine needs to be put into perspective. Only for resistance problems in zoonotic pathogens such as Salmonella serotypes, Campylobacter spp. and some enterococci can it be said with certainty that veterinary medicine carries a certain degree of responsibility (Franklin, 1999; Schwarz et al., 2001; White et al., 2001, Swartz, 2002).
REFERENCES


Christensen H., Angen O., Olsen J.E., Bisgaard M. 2004a. Revised description and classification of atypical isolates of *Pasteurella multocida* from bovine lungs based on genotypic characterization to include variants previously classified as biovar 2 of *Pasteurella canis* and *Pasteurella avium*. Microbiology 150: 1757-1767.

Christensen H., Bisgaard M., Aelbaek B., Olsen J.E. 2004b. Reclassification of Bisgaard taxon 33, with proposal of *Volucribacter psittaciicida* gen.nov., sp. nov. and *Volucribacter amazonae* sp. nov. as new members of the *Pasteurellaceae*. Int. J. Syst. Evol. Microbiol. 54, 813-818.


NCCLS-National Committee for Clinical Laboratory Standards. 2004. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; informational supplement, M31-S1, National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania, USA.


Chapter 1.2.
1.2. AIMS OF THE STUDY

The general aim of the present thesis was to study the occurrence of antimicrobial resistance in potential bovine pathogens belonging to the genera *Pasteurella* and *Mannheimia*. Since antimicrobial susceptibilities may vary among different bacterial species belonging to a certain genus and since substantial reclassifications have recently taken place within the bacterial family of *Pasteurellaceae*, it was necessary to develop a reliable identification method allowing to differentiate *Pasteurellaceae* up to (sub) species level.

The specific aims of the present thesis were:

1. To evaluate the usefulness of tRNA-intergenic spacer PCR for the species identification of bovine *Pasteurellaceae*.

2. To investigate the presence of tetracycline resistant subpopulations among *Pasteurellaceae* in the upper respiratory tract of healthy calves.

3. To compare the presence of different *Pasteurellaceae* in the upper respiratory tract from calves housed under different production types and their antimicrobial resistance patterns, with particular reference to tetracycline resistance.

During the execution of the aforementioned studies, we were faced with a severe and atypical outbreak of bovine pasteurellosis associated with a multi-resistant *Pasteurella* organism. A detailed report of this case, including the description of a novel plasmid-borne spectinomycin/streptomycin resistance gene, is also included in this thesis.
CHAPTER 2.

EXPERIMENTAL & OBSERVATIONAL STUDIES
CHAPTER 2.1.

TRNA-INTERGENIC SPACER PCR FOR THE IDENTIFICATION OF PASTEURELLA AND MANNHEIMIA SPP.

Boudewijn Catry, a Margo Baele, b Geert Opsomer, a Aart de Kruif, a Annemie Decostere, b Freddy Haesebrouck b


aDepartment of Reproduction, Obstetrics, and Herd Health,
bDepartment of Pathology, Bacteriology and Poultry Diseases,
Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium.
Chapter 2.2.
tRNA intergenic spacer PCR (tDNA-PCR) was evaluated for its effectiveness in differentiating *Pasteurella* and *Mannheimia* (sub)species predominantly of ruminant origin. For this purpose, 38 reference strains and 13 field isolates belonging to both genera were investigated. tDNA-PCR enabled discrimination of all *Pasteurella* species tested (*Pasteurella (P.) aerogenes*, *P. avium*, *P. canis*, *P. lymphangitidis*, *P. multocida*, *P. trehalosi*). For the differentiation of the subspecies of *P. multocida*, an additional dulcitol reaction was required. Two of the five so-far-defined *Mannheimia* species, *M. granulomatis* and *M. varigena*, had a distinct fingerprinting profile. The remaining three phylogenetically highly related species (*M. haemolytica*, *M. glucosida*, and *M. ruminalis*) clustered together. Nevertheless, *M. ruminalis* is non-haemolytic, and *M. haemolytica* and *M. glucosida* can be differentiated on the basis of two additional phenotypic characteristics (β-glucosidase and aesculin hydrolysis). In conclusion, tDNA-PCR is a useful tool in differentiating organisms belonging to the genera *Pasteurella* and *Mannheimia*. 
Chapter 2.2.

1. INTRODUCTION

The trehalose negative [*Pasteurella* *haemolytica* complex was recently transferred into the new genus *Mannheimia*, with *Mannheimia haemolytica* (*Pasteurella* *haemolytica* Newsom and Cross 1932) being the type species, and containing at least four other species (Angen *et al.*, 1999a). *Pasteurella* and *Mannheimia* species are present on mucous membranes of different warm-blooded animals, predominantly in the upper respiratory tract and the gastrointestinal tract. There they can act as primary or opportunistic pathogens causing a wide range of diseases leading to an important decline in livestock production (Quinn, 1994; Ackermann and Brogden, 2000). In ovine and bovine pneumonia, which is the paramount cause of losses to the sheep and cattle industry worldwide, *M. haemolytica* and *P. multocida* are among the most important bacterial pathogens involved (Liu *et al.*, 1999; Ackermann and Brogden, 2000; Hunt *et al.*, 2000; Catry *et al.*, 2002).

The limited reliability of serotyping in both *Pasteurella* (Wilson *et al.*, 1993) and *Mannheimia* species (Angen *et al.*, 1999a; b), the confusing results in phenotyping *P. multocida*-like strains (Hunt Gerardo *et al.*, 2001) and the influence of culture conditions on phenotypic characteristics of both *P. multocida* (Jacques *et al.*, 1994) and *M. haemolytica* (Rowe *et al.*, 2001), illustrate the complexity of the identification of these organisms. Extensive polyphasic studies, combining extensive phenotyping and laborious molecular methods such as ribotyping, multilocus enzyme electrophoresis, 16S rRNA sequencing and DNA-DNA hybridisation, have been required to organise both genera (Mutters *et al.*, 1985a; Sneath and Stevens, 1990; Angen *et al.*, 1997; 1999a). In this respect, an accurate and rapid molecular identification tool would improve studies on pathogenesis, epidemiology and antimicrobial resistance within the genera *Pasteurella* and *Mannheimia*.

Most PCR-based assays that have been used for the differentiation of *Pasteurella* spp. are focused on *P. multocida* and its subspecies (Hunt *et al.*, 2000; Hunt Gerardo *et al.*, 2001; Petersen *et al.*, 2001; Miflin and Blackall, 2001; Amonsin *et al.*, 2002; Chen *et al.*, 2002; Lainson *et al.*, 2002). These studies mainly covered isolates from human, canine, feline, porcine and avian origin, whereas markedly less attention has been paid to ruminant field isolates. In addition, no PCR-fingerprinting technique has been published in which the recently defined species of the novel genus *Mannheimia* (Angen *et al.*, 1999a) were included.
tRNA-intergenic spacer PCR (tDNA-PCR) (Welsh and McClelland, 1991) is a rapid molecular identification method that has been applied for species differentiation in *Acinetobacter* spp., *Lactobacillus* spp., *Listeria* spp. and several Gram-positive cocci (Baele *et al.*, 2000; 2001; 2002). This PCR fingerprinting method is based upon the amplification of spacers between the transfer RNA genes, using consensus primers complementary to the highly conserved edges of the tRNA genes and directed outwardly. Capillary electrophoresis of the resulting PCR fragments enhances the discriminatory power. The purpose of this study was to determine whether tDNA-PCR is a reliable method for the differentiation of the clinically important bacteria belonging to the genera *Pasteurella* and *Mannheimia*, with special reference to ruminant isolates.

2. MATERIALS AND METHODS

2.1. Bacterial strains

The bacterial collection used in this study consisted of 38 reference strains belonging to 6 species of the genus *Pasteurella* and 5 species within the genus *Mannheimia*. Herein 11 type strains and a so far undefined taxon belonging to the former *[Pasteurella] haemolytica*-complex were included (*Mannheimia* spp. CCUG 38464; biovar 8A). The reference strains and their origin are listed in Table 1. The selection represents most important animal and human pathogens that are, so far, well defined within both genera (Mutters *et al.*, 1985a;b; Sneath and Stevens, 1990; Bisgaard *et al.*, 1991; Quinn, 1994; Angen *et al.*, 1999a). This series was extended with 10 and 3 field strains, presumptively identified as *[Pasteurella] haemolytica* and *P. multocida*, respectively, according to Quinn (1994). These field strains were isolated in Belgium during the period 1999-2000, from cattle (tracheal washings) suffering from respiratory disease.

2.2. Phenotypic identification

Colony morphology was evaluated on sheep blood agar (Columbia, Oxoid, Hampshire, UK) after overnight incubation in a 5% CO$_2$-enriched environment at 37°C. A Gram stain was performed. The following biochemical traits were determined: catalase and oxidase production, motility, indole production (dimethylaminobenzaldehyde), ornithine decarboxylase (ODC) (Motility Indole Ornithine Medium, Beckton Dickinson, Franklin Lakes, NJ, USA) production, and ability to ferment glucose, lactose and sucrose and production of hydrogen sulphide by inoculation in TSI (triple sugar iron) (Quinn, 1994).
Table 1. Reference strains of *Pasteurella* and *Mannheimia* used in the present study

<table>
<thead>
<tr>
<th><strong>Species</strong></th>
<th><strong>Source</strong></th>
<th><strong>Reference number</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pasteurella multocida ss. multocida</em></td>
<td>Cat</td>
<td>CCUG 43536</td>
</tr>
<tr>
<td></td>
<td>Human blood</td>
<td>CCUG 37250</td>
</tr>
<tr>
<td></td>
<td>Porcine</td>
<td>NCTC 10322&lt;sup&gt;T&lt;/sup&gt; (LMG 2851&lt;sup&gt;T&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>Bovine</td>
<td>NCTC 3195 (LMG 4222)</td>
</tr>
<tr>
<td><em>Pasteurella multocida ss. gallicida</em></td>
<td>Bovine</td>
<td>CCUG 17978&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Duck</td>
<td>CCUG 17980</td>
</tr>
<tr>
<td></td>
<td>Swine</td>
<td>CCUG 26980</td>
</tr>
<tr>
<td><em>Pasteurella multocida ss. septica</em></td>
<td>Human blood</td>
<td>CCUG 38669</td>
</tr>
<tr>
<td></td>
<td>Human sepsis</td>
<td>CCUG 43535</td>
</tr>
<tr>
<td></td>
<td>Human wound</td>
<td>CCUG 33541</td>
</tr>
<tr>
<td></td>
<td>Human wound</td>
<td>CCUG 35887</td>
</tr>
<tr>
<td><em>Pasteurella avium</em></td>
<td>Chicken sinus</td>
<td>CCUG 12833&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Chicken</td>
<td>CCUG 26458</td>
</tr>
<tr>
<td><em>Pasteurella canis</em></td>
<td>Human</td>
<td>CCUG 895</td>
</tr>
<tr>
<td></td>
<td>Dog throat</td>
<td>CCUG 12400&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Human wound</td>
<td>CCUG 46325</td>
</tr>
<tr>
<td><em>Pasteurella aerogenes</em></td>
<td>Bovine bone</td>
<td>CCUG 27905</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>CCUG 27934</td>
</tr>
<tr>
<td><em>Pasteurella lymphangitidis</em></td>
<td>Bovine</td>
<td>CCUG 27188&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Pasteurella trehalosi</em></td>
<td>Lamb septicaemia</td>
<td>CCUG 27190&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Bovine lung</td>
<td>CCUG 37711</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>CCUG 35739</td>
</tr>
<tr>
<td></td>
<td>Septic calf</td>
<td>CCUG 46489</td>
</tr>
<tr>
<td><em>Mannheimia haemolytica</em></td>
<td>Sheep</td>
<td>CCUG 12392&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Calf pneumonia</td>
<td>CCUG 38454</td>
</tr>
<tr>
<td></td>
<td>Calf pneumonia</td>
<td>CCUG 43453</td>
</tr>
<tr>
<td><em>Mannheimia glucosida</em></td>
<td>Sheep</td>
<td>CCUG 28376</td>
</tr>
<tr>
<td></td>
<td>Ovine lung</td>
<td>CCUG 38457&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ovine nose</td>
<td>CCUG 38459</td>
</tr>
<tr>
<td><em>Mannheimia ruminalis</em></td>
<td>Ovine rumen</td>
<td>CCUG 38466</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>CCUG 38470&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ovine</td>
<td>CCUG 38471</td>
</tr>
<tr>
<td><em>Mannheimia varigena</em></td>
<td>Bovine lung</td>
<td>CCUG 38462&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Pig sepsis</td>
<td>CCUG 38472</td>
</tr>
<tr>
<td></td>
<td>Bovine udder</td>
<td>CCUG 38475</td>
</tr>
<tr>
<td><em>Mannheimia granulomatis</em></td>
<td>Deer lung</td>
<td>CCUG 34655</td>
</tr>
<tr>
<td></td>
<td>Bovine tongue</td>
<td>CCUG 38461</td>
</tr>
<tr>
<td></td>
<td>Bovine granuloma</td>
<td>CCUG 45422&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mannheimia</em> spp. biogroup 8A</td>
<td>Ovine</td>
<td>CCUG 38464</td>
</tr>
</tbody>
</table>

<sup>a</sup> CCUG: Culture Collection University of Göteborg, Sweden; NCTC: National Collection of Type Cultures, Colindale, UK; LMG: Belgian Coordinated Collection of Microorganisms culture collection (BCCMTM-LMG), Ghent University, Belgium. <sup>b</sup> A superscript T indicates the type strain.
For the *P. multocida* isolates, Diatabs® Diagnostic Tablets (Rosco, Taarstrup, Denmark) were used to investigate urease production, aesculin hydrolysis, \( \beta \)-galactosidase (ONPG), and fermentation of trehalose, L-arabinose, maltose, mannitol, D-xylose, and D-sorbitol. For each field strain, a suspension of fresh colonies was adjusted to McFarland standard 4 in phosphate buffered saline (PBS). Tablets were inserted under sterile conditions in 96 microtiter plates, and 0.25 ml of the bacterial suspension was added. The microtiter plates were incubated for 48 hours under atmospheric conditions at 37°C, and the different tests evaluated according to the manufacturer’s guidelines (available at www.rosco.dk) at 24 and 48 hours. Fermentation of dulcitol was investigated using Taxo® carbohydrate discs (BBL, Sparks, MD, USA). To tubes containing 2 ml of Phenol Red Broth, the inoculum was introduced after aseptically inserting a dulcitol containing disc. In each of the series tested, a negative control tube without inoculum was included. After incubation at 37°C, results were interpreted according to the colour change of the suspension at 4, 6, and 18 hours. All field strains were tested twice to assess reproducibility of the results. Differentiation at species and subspecies level was done according to Table 2. The following reference strains were included: *P. multocida* ss. *multocida* NCTC 10322\(^T\) (LMG 2851), *P. multocida* ss. *septica* CCUG 33541, and *P. multocida* ss. *gallicida* CCUG 17978\(^T\). In addition, we tested all eleven reference strains of *P. multocida* (Table 1) twice for sorbitol and dulcitol fermentation as described above.

Additional phenotyping of [*Pasteurella*] *haemolytica* field strains was performed as described by Angen et al. (2002). Briefly, following reactions were investigated using Diatabs® Diagnostic Tablets (Rosco): urease, aesculin hydrolysis, \( \beta \)-glucosidase (NPG), \( \alpha \)-fucosidase (ONPF), and fermentation of trehalose, L-arabinose, mannitol, and D-sorbitol. Testing was done as described above, with the exception that densities of bacterial suspensions exceeded McFarland standard 7.5. All strains were tested twice for reproducibility purposes. The differentiation scheme for *Mannheimia* species is presented in Table 3. Control strains used were *M. haemolytica* CCUG 12392\(^T\), *M. glucosida* CCUG 38457\(^T\), *M. varigena* CCUG 38462\(^T\), *M. ruminalis* CCUG 38470\(^T\), and *M. granulomatis* CCUG 38461.
### Table 2. Phenotypic characteristics used to separate the investigated *Pasteurella* organisms\(^a\)

<table>
<thead>
<tr>
<th>Test</th>
<th><em>P. aerogenes</em>(^b)</th>
<th><em>P. avium</em></th>
<th><em>P. canis</em></th>
<th><em>P. lymphangitidis</em></th>
<th><em>P. trehalosi</em></th>
<th><em>P. multocida ss. multocida</em></th>
<th><em>P. multocida ss. septica</em></th>
<th><em>P. multocida ss. gallicida</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolysis (ovine blood)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+(^d)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-(^d)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-(^d)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+(^d)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-(^d)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-(^d)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Sorbitol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+(^d)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+(^d)</td>
<td>-</td>
<td>+(^d)</td>
<td>-(^d)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+(^d)</td>
<td>+</td>
<td>+(^d)</td>
<td>-(^d)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aesculin</td>
<td>-</td>
<td>-</td>
<td>+(^d)</td>
<td>-(^d)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+(^d)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ß-galactosidase (ONPG)</td>
<td>d</td>
<td>-</td>
<td>+(^d)</td>
<td>-(^d)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>d</td>
<td>+</td>
<td>-</td>
<td>-(^d)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) Adapted from Mutters *et al.*, 1985a; Sneath and Stevens, 1990; Quinn, 1994; Fegan *et al.*, 1995. Rectangle presents the tests to differentiate the subspecies of *P. multocida*. \(^b\) *P. aerogenes* produces gas from glucose. \(^c\), +, positive; -, negative; d, + or -. \(^d\) Deviating strains exist.

### Table 3. Phenotypic characteristics used to separate the investigated [*P.*] haemolytica-like organisms\(^a\)

<table>
<thead>
<tr>
<th>Test</th>
<th>Defined species within the novel genus <em>Mannheimia</em> (M.)</th>
<th><em>M. haemolytica</em></th>
<th><em>M. glucosida</em></th>
<th><em>M. varigena</em></th>
<th><em>M. granulomatis</em></th>
<th><em>M. ruminalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolysis (ovine blood)</td>
<td>-(^b)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Sorbitol</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>-</td>
<td>d</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aesculin</td>
<td>-</td>
<td>+(^c)</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ß-fucosidase (ONPF)</td>
<td>+</td>
<td>+(^c)</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ß-glucosidase (NPG)</td>
<td>-</td>
<td>+</td>
<td>d</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>-</td>
<td>d</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>-(^c)</td>
</tr>
</tbody>
</table>

\(^a\) Previous classifications and original data according to Angen *et al.* (1999a; 2002). \(^b\), +, positive; -, negative; d, + or -. \(^c\) Deviating strains exist.
2.3. DNA preparation

Bacterial cells were grown overnight on sheep blood agar (Columbia, Oxoid) at 37°C in a 5% CO₂-enriched environment and checked for purity. A loopful of cells was suspended in 20 µl of lysis buffer (0.25% sodium dodecyl sulfate, 0.05 N NaOH) and heated at 95°C for 5 min. The cell lysate was spun down by brief centrifugation at 16,000 × g and diluted by adding 180 µl of distilled water. The cell debris was removed by centrifugation at 16,000 × g for 5 min. Supernatants were directly used as the template for PCR.

2.4. tDNA-PCR

PCR was carried out using the outwardly directed tRNA gene consensus primers T5A (5’ AGTCCGGTGCTCTAACCAACTGAG) and T3B (5’ AGGTCGCGGGTTCGAATCC), as described by Welsh and McClelland (1991). Reactions were carried out in a 10 µl volume containing 9.1 µl of High Fidelity Mix 1.1× (Invitrogen Life Technologies, Merelbeke, Belgium). Primers were added at a final concentration of 0.1 µM. Primer T3B consisted of a mixture of 1/5 fluorescent TET-labeled oligonucleotides and 4/5 nonlabeled oligonucleotides (Applied Biosystems, Foster City, CA, USA). A volume of 0.7 µl of sample DNA was added (the template was diluted 15 times). After 2 min at 94°C, reaction mixtures were cycled 30 times in a Perkin-Elmer Cetus 9600 thermocycler under the following conditions: 30 s at 94°C, 1 min at 50°C, and 1 min at 72°C. The final extension was 30 min at 72°C. Reaction vials were then cooled to 10°C and kept on ice until used in electrophoresis.

2.5. Capillary electrophoresis

Twelve µl of deionized formamide was mixed with 0.5 µl of an internal size standard mixture, containing 0.3 µl of the GS-400 High Density size standard and 0.2 µl of the GS-500 standard, which both have ROX-labeled fragments in the range of 50 to 500 bp (Applied Biosystems). One µl of tDNA-PCR product was added. The mixtures were denatured by heating at 95°C for 3 min and placed directly on ice for at least 15 min. Capillary electrophoresis was carried out using an ABI-Prism 310 Genetic Analyzer (Applied Biosystems) at 60°C, a constant voltage of 1.5 kV, and a more or less constant current of approximately 10 mA. Capillaries with a length of 47 cm and a diameter of 50 µm were filled with Performance Optimized Polymer 4. Electropherograms were normalized using Genescan Analysis software, version 2.1 (Applied Biosystems). The
fragment lengths were derived from the peak positions after interpolation with the peak positions of the size standard fragments.

2.6. Data analysis

tDNA spacer peak values (fragment lengths in base pairs) were obtained from the Genescan Analysis software in table form, and processed with the in-house software (available on request) described previously (Baele et al., 2000). Shortly, for all Pasteurella and Mannheimia species, a (sub)species-specific entry was constructed based upon the averages of the peak values in the tDNA-PCR fingerprints obtained after testing different strains of each (sub)species. Then, species entries were organised into a library in order to identify an unknown pattern of the field isolates. Similarities between patterns were calculated with the dbp (differential base pairs) coefficient, which is defined as the number of fragments in common between the unknown fingerprint and the species entry, divided by the total number of fragments of the species entry in the library (Baele et al., 2001). A peak position tolerance of 0.8 bp was used. A distance matrix was calculated with the in-house software and clustering analysis was done using UPGMA (Baele et al., 2001). For testing reproducibility, the majority of strains were analysed (PCR and electrophoresis) at least two times.

3. RESULTS

3.1. tDNA-PCR

By visual inspection of the electropherograms (Fig. 1) obtained by tDNA-PCR, it was possible to discriminate all Pasteurella species used in this study. Ten reference strains were tested three times, using different PCR-mixtures, different thermal cycling runs and different electrophoresis runs. The maximal range obtained for all peaks was 1.6 bp (base pairs), which is comparable with previous results (Baele et al., 2001). For use of the in-house software, a tDNA-PCR profile library (Table 4) was manually constructed, containing all reproducible fragment length values for each species tested. A dendrogram based on tDNA-PCR patterns illustrates the discriminatory power for the different Pasteurella species (Fig. 2). The P. multocida ss. multocida and ss. gallicida showed similar tDNA-PCR patterns, while P. multocida ss. septica had two extra peaks. M. haemolytica and M. glucosida strains, and the reference strain of Mannheimia biogroup 8A showed indistinguishable tDNA-PCR profiles. M. ruminalis strains showed similar
patterns but one fragment differed one base pair in length with *M. haemolytica*/*M. glucosida* (Table 4). *M. varigena* and *M. granulomatis* showed species-specific patterns.

![Figure 1](image_url). Figure 1. tDNA-PCR fingerprint patterns of *Pasteurella* species; e.g. *P. canis* CCUG 12400\(^T\) (top) and *P. avium* CCUG 12833\(^T\) (bottom). The x axis represents the fragment length in base pairs; the y axis represents the peak intensity.

### 3.2. Identification of field strains

The sorbitol and dulcitol fermentation tests enabled us to correctly delineate the eleven reference strains of *P. multocida* into the respective subspecies of *P. multocida* (rectangle in Table 2). A clear distinction between dulcitol fermenting and non-fermenting strains was however only possible after 18 hours of incubation and positive fermentations of sorbitol were rather weak. Results of biochemical tests revealed that the 3 field strains presumptively identified as *P. multocida* belonged to the subspecies *multocida*. This was in agreement with the result obtained by tDNA-PCR.

Based on the discriminatory properties of the *Mannheimia* species presented in Table 3, four field isolates were identified as *M. haemolytica*, two as *M. glucosida*, and two as *M. varigena*. The two remaining organisms (*Mannheimia* UT-MIC15 and *Mannheimia* UT-MIC117) were untypable. Strain UT-MIC15 was haemolytic, ODC negative, aesculin negative, β-glucosidase (NPG) positive, and fermented both arabinose and sorbitol. The other strain (UT-MIC117) was haemolytic, ODC negative, aesculin negative, β-glucosidase (NPG) positive, and fermented sorbitol. Additional testing for β-glucosidase (NPG) and aesculin of the reference strains of *M. haemolytica* and *M. glucosida* revealed affirmation of the species identification according to Table 3. Strain CCUG 38464 (*Mannheimia* spp. biovar 8A) was negative for both aesculine and β-glucosidase (NPG), though aesculine reaction was during retesting (3 times) once positive.
Table 4. Manually constructed tDNA-PCR library composed of organism specific entries that each consisting of tDNA spacer fragment lengths (base pairs)

<table>
<thead>
<tr>
<th>Species entry</th>
<th>tDNA spacer fragment lengths</th>
<th>Cluster(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasteurella multocida ss. multocida/ ss. gallicida</td>
<td>58.5,72.9,99.6,106.8</td>
<td>Pm II</td>
</tr>
<tr>
<td>Pasteurella multocida ss. septica</td>
<td>58.5,87.5,99.5,101.8,106,221.5</td>
<td>Pm I</td>
</tr>
<tr>
<td>Pasteurella avium</td>
<td>56.5,62.8,154.8,724.7,725.4</td>
<td>P. avium</td>
</tr>
<tr>
<td>Pasteurella canis</td>
<td>58.6,84.8,96.4,106,225.5</td>
<td>P. canis</td>
</tr>
<tr>
<td>Pasteurella aerogenes</td>
<td>58.5,75,81.5,88.91.5,92.5,199.2</td>
<td>P. aerogenes</td>
</tr>
<tr>
<td>Pasteurella lymphangitidis</td>
<td>59.4,149,3,210.3</td>
<td>P. lymphangitidis</td>
</tr>
<tr>
<td>Pasteurella trehalosi</td>
<td>58.5,77.2,82.5,88.93,185.5</td>
<td>P. trehalosi</td>
</tr>
<tr>
<td>Mannheimia haemolytica/glucosida/biogroup 8A</td>
<td>58.5,74.5,84.8,93,184.8</td>
<td>M UM II</td>
</tr>
<tr>
<td>Mannheimia ruminalis</td>
<td>58.5,74.5,82.8,184.8</td>
<td>M UM II</td>
</tr>
<tr>
<td>Mannheimia granulomatis</td>
<td>58.5,82,98.2,121.5,188,308</td>
<td>M. granulomatis (M III)</td>
</tr>
<tr>
<td>Mannheimia varigena</td>
<td>58.5,84,86.5,186</td>
<td>M. varigena (M IV)</td>
</tr>
</tbody>
</table>

\(^a\) Cluster names refers to the dendrogram presented in Fig. 2
Figure 2. Dendrogram obtained from tDNA-PCR fingerprints after similarity calculation with in-house software and clustering with UPGMA. The bar represents a distance of 10%.
The separation by means of tDNA-PCR of these 10 field isolates belonging to the genus *Mannheimia*, was in agreement with the results obtained with the reference strains (Fig. 2). Briefly, field strains identified as *M. glucosida*, *M. haemolytica*, and one untypable strain (UT-MIC117) gave indistinguishable fingerprinting profiles. The other untypable *Mannheimia* organism, had a distinct fingerprinting profile (UT-MIC15).

4. DISCUSSION

The generic arrangements within the family *Pasteurellaceae* have undergone considerable change in the last two decades (Mutters *et al.*, 1985a; b; Sneath and Stevens, 1990; Angen *et al.*, 1999a). Because delineation of *Pasteurella* species and subspecies (Mutters *et al.*, 1985; Muhairwa *et al.*, 2001) and proper identification of *[P.] haemolytica*-like organisms requires extensive phenotyping (Angen *et al.*, 2002), the discriminative power of tDNA-PCR for predominantly ruminant *Pasteurella* and *Mannheimia* spp. was evaluated in this study, using both reference and field strains. Since serotyping is considered not to be a reliable identification tool for *P. multocida* (Wilson *et al.*, 1993) and *[P.] haemolytica*-like strains (Angen *et al.*, 1999b; 2002), it was not performed in the present study.

The reference strains of the six *Pasteurella* species tested showed clearly different tDNA-PCR patterns (Fig. 1; Table 4), demonstrating that tDNA-PCR is a reliable method for differentiation of these species. For the discrimination of the three subspecies within *P. multocida*, tDNA-PCR clearly separated *P. multocida* ss. *septica* (cluster PmI) from *P. multocida* ss. *multocida* and ss. *gallicida* strains (cluster PmII). This is in complete agreement with the phylogenetic analysis by 16S rRNA gene sequencing as recently performed by different study groups (Kuhnert *et al.*, 2000; Petersen *et al.*, 2001; Chen *et al.*, 2002). To further differentiate *P. multocida* ss. *multocida* and ss. *gallicida*, dulcitol fermentation was used. Dulcitol fermentation has been attributed to be a stable marker in *P. multocida* isolates, in contrast with trehalose (Fegan *et al.*, 1995) and sorbitol (Hunt Gerardo *et al.*, 2001) fermentation. With the dulcitol fermentation test used in this study, a clear interpretation was only possible at 18 hours post incubation, although the manufacturer recommends 4 and 6 hours to be sufficient if the procedure in phenol broth is used. Nevertheless, we find the method used reproducible and accurate since consistent differences were seen in dulcitol fermentation (Table 2) of all eleven *P. multocida* reference strains (Table 1). From this, it can be concluded that dulcitol fermenting
organisms located in group PmII of the tDNA-PCR derived dendrogram (Fig. 2), can be identified as *P. multocida* ss. *gallicida*.

Within the genus *Mannheimia*, tDNA-PCR showed a clear differentiation for the reference strains of *M. varigena* and *M. granulomatis*, while the reference strains of the three other species (*M. haemolytica*, *M. glucosida*, and *M. ruminalis*) and the biogroup 8A strain of *Mannheimia* (CCUG 38464) clustered together (Fig. 2). By combination of extended phenotyping, 16S rRNA analysis, MLEE, ribotyping and DNA-DNA hybridizations, the phylogenetic relatedness of the new *Mannheimia* spp. within the former *[P.]* haemolytica-complex was established (Angen et al., 1997; 1999a). The discrimination of *Mannheimia* spp. by tDNA-PCR (Fig. 2) is in complete agreement with 16S rRNA analysis performed by Angen et al. (1999a). The final delineation of *M. haemolytica*, *M. glucosida* and *Mannheimia* spp. biogroup 8A was based upon DNA-DNA-hybridisations. Interestingly, these hybridisation studies were limited in number and characterised by a remarkable wide range (Angen et al., 1999a). Thus, to differentiate *Mannheimia* strains belonging to the species *M. haemolytica*, *M. glucosida*, *M. ruminalis* and biogroup 8A, three additional phenotypical characteristics are required as shown in Table 3: haemolysis, β-glucosidase (NPG) activity and aesculin hydrolysis. β-glucosidase, and to a lesser extent aesculin, have been attributed to be stable markers within the genus *Mannheimia* (Angen et al., 1997; 1999a). Within the genus *Mannheimia*, some unnamed taxa (biogroups 7, 8B, 8C, 9, 10) have been described that are both β-glucosidase and aesculin negative (Angen et al., 1999a) similar to *M. haemolytica*. These biogroups are however genetically not highly affiliated to the more tight group of *M. haemolytica*, *M. glucosida* and biogroup 8A. It is therefore not likely for these taxa to cluster within the former group by means of tDNA-PCR (cluster MI-MII in Fig. 2). In the present investigation, untypable strain UT-MIC15 was assigned to biogroup 10 (data not shown) and gave indeed a distinct fingerprinting profile (Fig. 2). More difficult is the differentiation between *M. haemolytica* and strain CCUG 38464 (biogroup 8A), because both present a negative β-glucosidase and aesculin test. However, strain CCUG 38464 (biogroup 8A) produced no pronounced zone of haemolysis, i.e. haemolysis was only visible after removal of colonies.

Interestingly, the three reference strains of *M. granulomatis* (CCUG 45422T, CCUG 38461, and CCUG 34655) revealed no haemolysis on the sheep blood agar used in this study. *M. granulomatis* is indeed considered to show no haemolysis on bovine blood agar (Angen et al., 1999a), but has been reported to be haemolytic using ovine blood (Ribiero et al., 1989). Therefore, surveys on identification of *Mannheimia* organisms
should be preceded by evaluating haemolysis reactions of the blood agar used by means of reference organisms.

Two of the field strains isolated from tracheal washings of cattle suffering from respiratory disease were identified as *M. glucosida*, two as *M. varigena*, and two were untypable. This demonstrates that several *Mannheimia* species, other than *M. haemolytica*, may also be involved in the bovine respiratory disease complex, which is in accordance with a recent Danish study (Angen *et al.*, 2002).

Previous investigations have demonstrated tDNA-PCR to be a reliable and rapid identification tool for large collections of bacteria, even without knowing the genus identity of the investigated organisms (Baele *et al.*, 2000; 2001; 2002). The reproducibility of the method and the interlaboratory exchangeability of the tDNA-PCR fingerprints have been reported in detail (Baele *et al.*, 2001; 2002). For the identification of genetically highly related organisms, it considerably narrows the array of biochemical tests required for a proper identification.

5. CONCLUSION

tDNA-PCR, expanded with a minimum of additional biochemical tests (dulcitol or β-glucosidase/aesculin), enables rapid discrimination of *Pasteurella* and *Mannheimia* (sub)species of human and veterinary importance. However, for both genera, a critical evaluation of the used media (haemolysis) and biochemical tests (e.g. dulcitol) by means of reference strains, is essential.

ACKNOWLEDGEMENTS

This work is supported by the Institute for the Promotion of Innovation by Science & Technology Flanders grant number IWT/SB/11134. The authors are very grateful for the excellent technical assistance of S. Haelterman and A. Van De Kerckhove. L.A. Devriese is acknowledged for delivering the field strains.

REFERENCES


Chapter 2.1.


CHAPTER 2.2.

ANTIMICROBIAL RESISTANCE IN PASTEURELLA AND MANNHEIMIA SPP. OF CALVES

2.2.1. Detection of tetracycline resistant and susceptible Pasteurellaceae in the nasopharynx of loose group housed calves

2.2.2. Variability in acquired resistance of Pasteurella and Mannheimia isolates from the nasopharynx of calves with particular reference to different herd types

2.2.3. Tet(L)-mediated tetracycline resistance in bovine Pasteurella and Mannheimia isolates

2.2.4. Fatal peritonitis in calves caused by a multi-resistant P. Multocida capsular type F

2.2.5. A novel spectinomycin/streptomycin resistance gene, ada14, from P. Multocida
DETECTION OF TETRACYCLINE RESISTANT AND SUSCEPTIBLE PASTEURELLACEAE IN THE NASOPHARYNX OF LOOSE GROUP HOUSED CALVES

Boudewijn Catry\textsuperscript{a}, Annemie Decostere\textsuperscript{b}, Stefan Schwarz\textsuperscript{c}, Corinna Kehrenberg\textsuperscript{c}, Aart de Kruif\textsuperscript{a}, Freddy Haesebrouck\textsuperscript{b}

Veterinary Research Communications 2005, in press.

\textsuperscript{a} Department of Reproduction, Obstetrics and Herd Health, Department of Pathology, Bacteriology, and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium

\textsuperscript{b} Institut für Tierzucht, Bundesforschungsanstalt für Landwirtschaft (FAL), 31535 Neustadt-Mariensee, Germany
Chapter 2.2.1.
ABSTRACT

The aim of the present study was to determine which Pasteurella and Mannheimia species are present in the upper respiratory tract of healthy calves with no history of antimicrobial treatment prior to sampling. The presence of subpopulations of tetracycline resistant Pasteurellaceae was also investigated. Therefore, nasal swabs from 61 loose group housed, 1 to 4 months old, clinically healthy calves from 16 dairy herds were inoculated aerobically on a selective medium (Columbia agar with 5% ovine blood and 16 mg/L bacitracin) with or without 4 mg/L oxytetracycline (OTC). A total of 43 strains belonging to the family Pasteurellaceae were isolated from 38 calves (62.3 %) out of 13 herds (81.3%). The most predominant organisms were Pasteurella (P.) multocida subsp. multocida (57.4%), Mannheimia (M.) varigena (4.9%), and M. haemolytica (3.2%). Growth of Pasteurellaceae on the OTC-containing medium was only seen with samples from two herds (6 animals; 9.8%), and in only one farm this proved to be an OTC-resistant subpopulation. MIC determinations by means of agar dilution confirmed a low prevalence of OTC-resistant Pasteurellaceae, with overall MIC$_{50}$ and MIC$_{90}$ values of 0.25 and 32 mg/L, respectively. These data do not support the hypothesis that the relative high frequency of tetracycline resistant P. multocida isolates from fatal cases of bovine respiratory disease is related to the presence of minor tetracycline resistance subpopulations within this species.
INTRODUCTION

Bovine respiratory disease (BRD) has been attributed to be the syndrome having the highest morbidity and mortality in calves (DeRosa et al., 2000). Mannheimia (M.) haemolytica and Pasteurella (P.) multocida, both belonging to the family Pasteurellaceae, are known to act as opportunistic pathogens in the BRD-complex (Quinn et al., 1994; Angen et al., 1999; Highlander, 2001). Older studies have shown that both species are present in the upper respiratory tract of healthy and diseased calves (Allen et al., 1991). The Pasteurellaceae recently underwent several reclassifications (Angen et al., 1999; Blackall et al., 2002; Angen et al., 2004; Christensen et al., 2004), with the introduction of the new genus Mannheimia (Angen et al., 1999) being the most substantial in respect of BRD. Since then, no surveys have been conducted in order to describe the presence of these new species within the nasopharynx of healthy calves.

Antimicrobial therapy is currently the most effective preventive and curative therapy for BRD (Schwarz and Chaslus-Dancla, 2001). Nevertheless, antimicrobial resistance is widespread among these opportunistic respiratory pathogens with tetracycline resistance being one of the most frequently reported resistance properties (Kehrenberg et al., 2001; Schwarz and Chaslus-Dancla, 2001). The bimodal minimum inhibitory concentration (MIC) distributions for oxytetracycline (OTC) found in both P. multocida and M. haemolytica (Mevius and Hartman, 2000) make resistant strains clearly distinguishable from susceptible strains. Interestingly, isolates from fatal cases (necropsy) of BRD harbour more often antimicrobial resistance determinants (Devriese et al., 1987; Mevius and Hartman, 2000) as compared to isolates retrieved from acute cases of BRD prior to therapy (Catry et al., 2002). From an epidemiological and therapeutic perspective, it is interesting to know whether this discrepancy is due to the presence of undetected resistant subpopulations already present prior to therapy. Following therapy, these subpopulations might either be selected and/or pass their resistance genes through horizontal gene transfer into the predominant pathogenic population (Catry et al., 2003; Sørum and Sunde, 2001). In case of horizontal gene transfer, plausible pathways are conjugation and mobilization of plasmids carrying resistance genes (Schwarz and Chaslus-Dancla, 2001).

The aim of the present study was to determine which Pasteurella and Mannheimia species are present in the upper respiratory tract of 1- to 4-months old calves with no history of antimicrobial treatment prior to sampling. The presence of subpopulations of
Tetracycline resistant subpopulations

tetracycline resistant Pasteurellaceae was also investigated.

MATERIALS & METHODS

Animals and Sampling

A total of 61 Holstein, East Flemish and mixed breed 1- to 4-months old calves were examined. They originated from 16 Belgian herds (A-P) and were loose group housed. From each herd, all calves within the age category and without a history of antimicrobial therapy prior to sampling (30 days) were included in the study. On two farms, the calves were vaccinated against M. haemolytica, parainfluenza-3 virus and bovine respiratory syncytial virus (Bovipast RSP®, Intervet) at 3 and 7 weeks of age (Table I). Sampling was performed in the period December 2002 – March 2003, which is the season with the highest incidence of BRD in the region. Prior to sampling, the nostril was disinfected using alcohol 90%. A sterile plastic swab was introduced in the nasal cavity (dorsal conchae, 15 cm depth) and rotated 360°. The sampling was performed with ethical committee approval. After sampling, each swab was inserted into a transport medium (Venturi Transsystem®, Copan) and cooled (4-7°C) during transport. Bacteriological investigations were set up within 2 hours after sampling.

Media

All nasal swabs were inoculated on Columbia blood agar (Oxoid) with 5% sheep blood and 16 mg/L bacitracin (Alpha Pharma) (BAC-agar), and on BAC-agar to which 4 mg/L OTC (Sigma) was added (BACOTC-agar). The latter medium was used for the isolation of tetracycline resistant strains. Plates were stored cooled (< 10°C) and used within three days after preparation.

To investigate a possible growth inhibition and change in colony morphology of Pasteurella and Mannheimia organisms on the BAC-agar, the growth of 3 P. multocida (CCUG (Culture Collection University of Göteborg, Sweden) 43536, CCUG 17978, CCUG 43535) and 3 M. haemolytica (CCUG 12392; CCUG 38454; CCUG 43453) reference strains was compared with that on Columbia agar (Oxoid) to which 5% sheep blood was added (blood agar). Briefly, for each strain 10-fold dilutions from a suspension corresponding to a density of 10^8 organisms per mL were plated on both media. Growth intensity (measured as CFU/mL) of appropriate dilutions were compared after 24 h of aerobic incubation at 37°C by measuring the relative growth-supporting ability, which was
determined as the logarithm of the ratio of the number of colonies on the control medium (blood agar) to the number of colonies on the BAC-medium from equivalent dilutions (Holm et al., 1987). Colony morphology was evaluated by visual inspection.

Bacteriological identification

Bacteria grown aerobically at 37°C were selected by colony morphology after incubation for 24h and 48h, followed by subculturing for another 24h on Columbia sheep blood (5%) agar (Oxoid). Colonies resembling Pasteurella or Mannheimia isolates were checked for purity, morphology was re-evaluated and bacteria were tentatively designated as Pasteurella or Mannheimia (Quinn et al., 1994; Catry et al., 2004a). The identification of the organisms up to (sub)species level was molecularly confirmed by means of tDNA-PCR and a maximum of two biochemical tests as described earlier (Catry et al., 2004a). In case of conflicting results for presumptively identified Mannheimia organisms by aforementioned methods, identification was based on phylogenetic relatedness through 16S rRNA sequencing (Vancanneyt et al., 2004) with following reference strains: Mannheimia haemolytica (AF060699), M. glucosida (AF053891 and AF053889), M. ruminalis (AF053900), M. varigena (AF053893), Mannheimia sp. HPA121 (AF053898), Mannheimia sp. R19.2 (AF053894), and Bisgaard Taxon 16 (AF224294). Sequences were determined by using an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Phylogenetic analysis was done by using the GENEBASE program (Applied Maths). Pairwise alignment homologies were calculated and a dendrogram was constructed by using the neighbour-joining method. Further details of this procedure are described elsewhere (Vancanneyt et al., 2004). If no Pasteurella or Mannheimia species were isolated, the bacteriological investigation was considered negative.

Antimicrobial susceptibility testing

Each identified Pasteurella or Mannheimia organism was tested for susceptibility to OTC through an agar dilution method (NCCLS, 2002) by means of the direct inoculation method. Briefly, overnight colonies were suspended in a sterile 0.9% NaCl solution and adjusted to 0.5 McFarland (Densimat, BioMérieux, France), whereafter a 1/10 dilution of these suspensions was spot-inoculated with a Steers type replicator (20 strains per plate) on Mueller Hinton II agar (Beckton Dickinson, France) supplemented with 5% sheep blood and containing two fold dilutions (range: 0.06-128 mg/L) of OTC (Sigma). Plates were incubated aerobically for 24 h at 37°C. The minimum inhibitory concentration
(MIC) was determined as the lowest concentration that inhibited visible growth. *Escherichia coli* ATCC 25922 was used as internal control organism. Interpretive criteria to determine resistance, intermediate resistance, and susceptibility were based upon breakpoints for “organisms other than streptococci” retrieved from NCCLS recommendations (NCCLS, 2002). Overall MIC$_{50}$ and MIC$_{90}$ data were calculated as the lowest concentrations of the antimicrobials that inhibited growth of 50% or 90%, respectively, of the organisms.

**RESULTS**

*Recovery efficiency from selective agar*

The mean relative growth supporting ability for the 6 reference strains on the BAC-agar was 0.06 (SD = 0.15), suggesting no substantial inhibition of *Pasteurella* and *Mannheimia* organisms on the selective medium compared to blood agar. Colony morphology of all strains was similar on BAC-agar and the control medium.

*Bacteriological investigations and antimicrobial resistance analysis*

On the BAC-medium a total of 42 *Pasteurellaceae* were isolated from 38 animals (62.3%) originating from 13 herds (81.3%). By means of tDNA-PCR and phenotyping, 3 haemolytic organisms were presumptively identified as untypable *Mannheimia* species. By means of their 16s rRNA sequence, 2 of the latter revealed to be *M. haemolytica* (sequence similarities ≥ 99%), while 1 strain could not be assigned to any of the recognised species within the genus *Mannheimia*. Definitive bacteriological results are presented in Table I. *P. multocida* subsp. *multocida* was the most frequently isolated organism, with a detection rate of 68.8 % at herd level (11/16) and 57.4% at animal level (35/61). In two of the latter 35 isolations, other species belonging to the *Pasteurellaceae* were simultaneously retrieved from the same animal on the BAC-medium (Table I).

On the BACOTC-medium, tetracycline resistant *Pasteurellaceae* were detected in 9.8% (6/61) calves originating from 2 farms (12.5 %). In one of these farms, all 5 calves gave a positive culture for *P. multocida* subsp. *multocida* with a MIC for OTC of 32 mg/L.
Table I. Bacteriological findings and minimum inhibitory concentration (MIC) ranges of oxytetracycline per herd.

<table>
<thead>
<tr>
<th>Herd</th>
<th>No. of calves</th>
<th>No. of calves positive for</th>
<th>MIC range for OTC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. multocida</td>
<td>P. canis</td>
<td>P. trehalosi</td>
<td>M. haemolytica</td>
</tr>
<tr>
<td>A</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>5^d</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>G^+</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>J</td>
<td>4</td>
<td>3</td>
<td>1^d,e</td>
</tr>
<tr>
<td>K</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>L^+</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>5</td>
<td>3</td>
<td>1^e</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>P</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total (%)</td>
<td>61</td>
<td>35 (57.4 %)</td>
<td>1 (1.6 %)</td>
</tr>
</tbody>
</table>

^a^+^ indicates that calves on the respective farms were vaccinated against *Mannheimia haemolytica*. ^b^ Minimum inhibitory concentration (MIC) for oxytetracycline (OTC). ^c^All *P. multocida* isolates were subspecies *multocida*. ^d^Strains with resistance to OTC based upon both susceptibility testing and growth on tetracycline containing selective medium. ^e^isolated together with *P. multocida* from one animal.

Table II. Distribution of minimum inhibitory concentrations (MIC, mg/L) of oxytetracycline for 37 *Pasteurella* and 6 *Mannheimia* isolates from the nasopharynx of calves.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Number of isolates showing a MIC of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤0.06</td>
</tr>
<tr>
<td>P. canis</td>
<td>-</td>
</tr>
<tr>
<td>P. multocida supers. multocida</td>
<td>-</td>
</tr>
<tr>
<td>P. trehalosi</td>
<td>-</td>
</tr>
<tr>
<td>M. haemolytica</td>
<td>-</td>
</tr>
<tr>
<td>M. varigena</td>
<td>-</td>
</tr>
<tr>
<td>Mannheimia spp.</td>
<td>-</td>
</tr>
</tbody>
</table>

Applied breakpoint used to define susceptibility was ≤4 mg/L (NCCLS, 2002).
For these animals identical results were obtained with the BAC-medium without OTC. By visual inspection, the \textit{P. multocida} colonies on both media for these 5 animals were approximately the same in number. On the other farm, a single tetracycline resistant \textit{P. canis} (MIC 32 mg/L) colony was exclusively detected on the BACOTC-medium (Table I). In addition, this strain was indole positive and therefore categorised as \textit{P. canis} biovar 1.

The MIC-distribution for OTC of the isolated \textit{Pasteurella} and \textit{Mannheimia} organisms is given in Table II. Herein, in accordance with the results obtained from the selective BACOTC-medium, 5 \textit{P. multocida} isolates and 1 \textit{P. canis} strain were substantially less susceptible to OTC (MICs: 32mg/L). The bimodal distribution (Table II) clearly discriminated between the 37 susceptible (≤4 mg/L) and the 6 resistant (≥16 mg/L) strains. The overall MIC\textsubscript{50} and MIC\textsubscript{90} values of the 43 \textit{Pasteurellaceae} for OTC were 0.25 and 32 mg/L, respectively. In case all isolates from a farm were considered susceptible to OTC (MIC ≤4mg/L), the range of MICs for OTC within a certain herd was limited (Table I). The only exception was one herd (M) in which four different species were isolated. Herein, the obtained MICs were 0.125, 0.5, and 1 mg/L for three \textit{P. multocida} isolates, two \textit{Mannheimia} organisms, and a single \textit{P. trehalosi} strain, respectively.

**DISCUSSION**

The overall high detection rate of \textit{P. multocida} (57.4\%) in the nasopharynx of apparently healthy cattle is in contrast with other reports where it was only found in 0.5\% out of 971 (Allan \textit{et al}., 1985), and 0\% out of 40 (Barbour \textit{et al}., 1997) nasal swabs. This is likely due to the disinfection of the nostril and selective medium used in the present study, which minimised contamination of the samples by organisms other than \textit{Pasteurellaceae}. Allen \textit{et al} (1991), who also used a more stringent decontamination protocol, isolated \textit{P. multocida} in 46.7\% of 60 apparently healthy calves. However, in the latter study a higher number of haemolytic \textit{Mannheimia} strains was isolated (31.6\%) compared to our results (9.8 \%), which in part might be due to the fact that on two herds the animals were vaccinated against \textit{Mannheimia haemolytica}. Based on the present and previous results (Blackall \textit{et al}., 2002; Catry \textit{et al}., 2004b), \textit{M. varigena} is suspected to be -similarly to \textit{M. haemolytica}- an opportunistic bovine pathogen present in the upper respiratory tract.

\textit{P. multocida} organisms can be subdivided into three subspecies (subsp.), namely subsp. \textit{multocida}, subsp. \textit{septica}, and subsp. \textit{gallicida} (Catry \textit{et al}., 2004a). In agreement
with the present data, bovine *P. multocida* isolates usually belong to the subspecies *multocida* (Christensen et al., 2004). Christensen et al. (2004) also demonstrated by extended molecular techniques that the indole negative variants of *P. avium* and *P. canis* recovered from the bovine respiratory tract should be reclassified within the species *P. multocida*. Here, we report an indole positive *P. canis* (biovar 1) strain, which has hitherto been unreported in relation to the bovine respiratory tract.

The 5 tetracycline resistant *P. multocida* strains originated from the same herd, thus it is likely that they are clonal in nature. One tetracycline resistant *P. canis* strain was isolated on another herd. According to the farmers all (6) calves in which tetracycline resistant *Pasteurellaceae* were isolated did not receive antimicrobials 30 days prior to sampling. A possible explanation for the presence of these tetracycline resistant organisms might be a former selection pressure in combination with the well recognised persistence of tetracycline resistance genes (Catry et al., 2003). Horizontal transfer of resistance genes between ecologically related bacteria as has been suggested to have taken place between *Pasteurella aerogenes* and *Enterobacteriaceae* in the intestinal tract of swine for the *tet(B)* gene (Kehrenberg and Schwarz, 2001) can also not be excluded.

In the present study, tetracycline resistant strains were nevertheless rarely seen which is in accordance with a previous report on pathogenic *Pasteurellaceae* of calves housed under similar conditions in the same region (Catry et al., 2002). Fatal cases of BRD have been often related to the presence of tetracycline resistant *P. multocida* and *M. haemolytica* organisms (Devriese et al., 1987; Mevius and Hartman, 2000). One hypothesis is that tetracycline resistant subpopulations are present at the beginning of the disease, and consequently become the predominant organisms or spread their resistance genes during the course of the disease. Such resistant subpopulations might be overlooked during routine bacteriology, based on a standard investigation of a purified culture of one colony. From the cultures of the BAC-medium, we were unable to detect the presence of tetracycline resistant subpopulations within a certain herd. With the exception of one *P. canis* strain, no improvement of this detection was found using the tetracycline containing medium. Although we only used one concentration of OTC (4mg/L) in the latter medium, it is unlikely that the sensitivity of the method might have been improved by further expanding the tetracycline dilution scheme. Indeed, the within-herd range of the MICs for tetracycline still was narrow and it has been reported that resistant *P. multocida* and *M. haemolytica* strains have MICs between 32 and 256 mg/L, with no correlation between the MIC of tetracycline and the genomic localisation, nor the identity of the underlying resistance
Tetraacycline resistant subpopulations

In conclusion, tetracycline susceptible *P. multocida* isolates were the most frequently isolated bacteria in the nasopharynx of loose group housed and apparently healthy calves. Our data do not support the hypothesis that the relatively high frequency of tetracycline resistant *P. multocida* isolates from fatal cases of BRD is related to the presence of minor tetracycline resistance subpopulations within this species.

**ACKNOWLEDGEMENTS**

This work is supported by the Institute for the Promotion of Innovation by Science & Technology Flanders (Grant n° IWT/SB/11134). The authors are very grateful for the excellent technical assistance of E. Defré, S. Haelterman and V. Nöding. T. Raeymaekers is acknowledged for his help in collecting the samples and raw data.

**REFERENCES**


Chapter 2.2.1.


Christensen H., Angen O., Olsen J.E., Bisgaard M. 2004. Revised description and classification of atypical isolates of Pasteurella multocida from bovine lungs based on genotypic characterization to include variants previously classified as biovar 2 of Pasteurella canis and Pasteurella avium. Microbiology, 150, 1757-1767.


CHAPTER 2.2.2.

VARIABILITY IN ACQUIRED RESISTANCE OF PASTURELLA AND MANNHEIMIA ISOLATES FROM THE NASOPHARYNX OF CALVES WITH PARTICULAR REFERENCE TO DIFFERENT HERD TYPES

Boudewijn Catry, Freddy Haesebrouck, Sarne De Vliegher, Bianca Feyen, Mia Vanrobaeys, Geert Opsomer, Stefan Schwarz, Aart de Kruif

Microbial Drug Resistance 2005, in press.

"Department of Reproduction, Obstetrics and Herd Health,
Department of Pathology, Bacteriology, and Poultry Diseases,
Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium
Flanders Animal Health Service, 8820 Torhout, Belgium
Institut für Tierzucht, Bundesforschungsanstalt für Landwirtschaft (FAL),
31535 Neustadt-Mariensee, Germany
Chapter 2.2.2.
ABSTRACT

To measure the level of antimicrobial resistance in potential bovine respiratory pathogens at different production types, nasal swabs were collected from 57 calves of 13 dairy herds, 150 calves of 9 beef cattle herds, and 289 calves of 5 high density veal calf herds and investigated for the presence of Pasteurellaceae. All calves were less than 6 months old. Susceptibilities of the Pasteurella and Mannheimia isolates to eight antimicrobials were determined using an agar dilution method. Pasteurella multocida (37.3%) and haemolytic Mannheimia organisms (M. haemolytica sensu lato) (6.3%) were the most frequently detected organisms. The overall prevalence of isolates resistant to at least one antimicrobial tested (N isolates/total x 100) from the dairy, beef, and veal calves were 17.6% (6/34), 21.9 % (14/64), and 71.9 % (64/89), respectively. In isolates obtained on the veal calf herds, acquired resistance to ampicillin, oxytetracycline, potentiated sulfonamides, gentamicin, tilmicosin and enrofloxacin was frequently present and 32.6 % of these isolates were resistant to more than two of the tested antimicrobials. Resistance to ceftiofur and florfenicol was not detected. A substantial within-herd variability of species diversity and resistance profiles among isolates belonging to the genera Pasteurella and Mannheimia was found among the isolates of the veal calf farms.
INTRODUCTION

*Pasteurella multocida* and *Mannheimia haemolytica* can be present in the upper respiratory tract of healthy calves. Under stress conditions, such as transport, crowding and viral or *Mycoplasma* infections, these organisms can act as invaders of the lower respiratory tract causing bovine respiratory disease (BRD), also known as enzootic bronchopneumonia, or bovine pasteurellosis. This disease, characterised by severe dyspnoea, loss of appetite, fever, and nasal discharge, is attributed to the syndrome having the highest morbidity and mortality in calves. Due to an often ineffective vaccination, the most common and effective therapy consists of the administration of antimicrobial drugs. Unfortunately, resistance has been frequently reported in the aforementioned *Pasteurellaceae* for a wide range of antimicrobial agents approved for the treatment of BRD.

The antimicrobial regimens applied on dairy farms, beef cattle farms, and veal calf farms largely differ with respect to product, dose, duration, and formulation. In this regard, a herd type-related selection pressure, and consequently a different level of antimicrobial resistance can be expected.

The aim of the present study was to compare the occurrence of *Pasteurellaceae* and their antimicrobial resistance profiles in the upper respiratory tract of healthy calves from dairy, beef, and veal calf farms. In addition, attention has been paid to the recent reclassifications of relevant bovine species within the genera *Mannheimia* and *Pasteurella*.

MATERIALS & METHODS

Animals and Sampling

The study was conducted between November 2002 and July 2003. A total of 496 healthy calves aged between 1 and 6 months originating from 27 Belgian farms were included in the study: 57 Holstein calves from 13 dairy herds, 150 Belgian White Blue calves from nine beef cattle herds, and 289 calves of various breeds from five veal calf herds (Table 1). Approximately fifty percent of all calves within the age category present at the moment of investigation on the dairy and beef farms were sampled. If less than five animals meeting the age inclusion criterion were present on the latter farms at the moment of sampling, all calves were included. On the veal calf farms only 20% of the animals were randomly sampled.
Calves at two dairy farms and at three beef farms had been vaccinated with an inactivated vaccine against *Mannheimia haemolytica*, parainfluenza-3 virus and bovine respiratory syncytial virus (Bovipast RSP®, Intervet). The calves on the dairy farms were loosely housed in pens, and no antimicrobial drugs had been administered ca. 30 days prior to sampling. The majority of the beef calves were weaned and loosely housed in pens (six farms) or in individual igloo-boxes (two farms), while the calves suckled in one farm. Antimicrobials had been administered in only a minority of calves from the beef herds. On the veal calf farms, predominantly male Holstein Friesian calves from two weeks of age on were housed in individual wooden straw-bedded boxes at high population density. They were fed a milk replacer diet twice a day supplemented with antimicrobials (tetracyclines, tylosin, colistin, flumequine or the combination sulfonamides-trimethoprim) for the first 6 up to 41 days. The majority of the veal calves were between 4 and 10 weeks of age at the moment of sampling. Prior to sampling, the nostril was disinfected using alcohol 90% (v/v). A sterile swab was introduced in the nasal cavity (dorsal conchae, 15 cm depth) and rotated 360°. After sampling, each swab was inserted into a transport medium (Venturi Transsystem®, Copan, Bovezza, Italy) and cooled (<10°C) during transport. Bacteriological investigations were set up within 48 h after sampling. The sampling was performed with approval of the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

**Bacteriology**

The primary isolation of bacteria was done by directly streaking each swab onto Columbia blood agar (Oxoid) to which 5% sheep blood and 15 mg/L bacitracin (Alpha Pharma, Nazareth, Belgium) was added. Bacteria grown aerobically at 37°C were selected by colonial morphology at 24 h and 48 h post incubation, followed by subculturing for another 24 h on blood agar. Colonies resembling *Pasteurella* or *Mannheimia* isolates were checked for purity, morphology was re-evaluated, and bacteria were tentatively designated as *Pasteurella multocida* or *Mannheimia haemolytica sensu lato* (synonym for *P. haemolytica*) according to Quinn *et al.*28 The identification of the organisms up to species level was done by phenotyping and molecularly confirmed by means of tRNA-intergenic spacer PCR as earlier described.12 In case of conflicting results by aforementioned methods for the presumptively identified *Mannheimia haemolytica sensu lato* isolates, final identification was based on phylogenetic relatedness through 16S rRNA sequencing33 with following reference strains: *Mannheimia*
Chapter 2.2.2.

haemolytica (AF060699), M. glucosida (AF053891 and AF053889), M. ruminalis (AF053900), M. varigena (AF053893), Mannheimia sp. HPA121 (AF053898), Mannheimia sp R19.2 (AF053894), and Bisgaard Taxon 16 (AF224294). Sequences were determined by using an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Phylogenetic analysis was done by using the GENEBASE program (Applied Maths). Pairwise alignment homologies were calculated and a dendrogram was constructed by using the neighbour-joining method. Further details of this procedure are described elsewhere.\(^\text{33}\) If no Pasteurella or Mannheimia species were isolated, the bacteriological investigation was considered negative.

**Antimicrobial susceptibility testing**

Susceptibility testing by the agar dilution method\(^\text{25}\) was performed by means of the direct inoculation method on Mueller Hinton II agar (Beckton Dickinson, Le Pont De Claix, France) supplemented with 5% sheep blood. Briefly, overnight colonies were suspended in a sterile 0.9% NaCl solution and adjusted to 0.5 McFarland (Densimat, bioMérieux, Marcy l'Etoile, France). Thereafter a 1/10 dilution of these suspensions was spot-inoculated with a Steers type replicator (20 strains per plate) and incubated aerobically for 24 h at 37°C. Following antimicrobials (range in mg/L) were tested by means of two-fold dilutions: ampicillin (0.03-128), cefiofur (0.03-128), oxytetracycline (0.06-128), gentamicin (0.06-128), enrofloxacin (0.03-64), tilmicosin (0.06-128), florfenicol (0.03-128), and the combination trimethoprim-sulfadimidine (0.06/1.14-128/2432). For the latter antimicrobial combination, Mueller Hinton II agar (Becton Dickinson) was supplemented with 5% lysed horse blood. All products were supplied by Sigma, with the exception of ceftiofur (Pfizer Animal Health, Puurs, Belgium). Minimum inhibitory concentration (MIC) endpoints were read as the lowest concentration of an antibacterial compound that completely inhibited macroscopically visible growth of the inoculum. *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC29212 were used as quality control organisms. Additionally, *Staphylococcus aureus* ATCC 29213 and reference strains belonging to the genus Mannheimia (*M. haemolytica* CCUG 12392, *M. glucosida* CCUG 38457, *M. ruminalis* CCUG 38470, *M. varigena* CCUG 38462, *M. granulomatis* CCUG 45422, and *Mannheimia* biogroup 8A CCUG 38464) were included in two assays for comparative purposes.
**Data analysis**

Interpretive criteria to discriminate susceptible, intermediate and resistant isolates for ceftiofur, tilmicosin, florfenicol, and enrofloxacin, were based on CLSI criteria.\(^{25,26}\) For ampicillin, the potentiated sulfonamides, tetracycline and gentamicin no Pasteurella- or Mannheimia-specific CLSI-breakpoints are currently available. For these compounds, the modality of distribution (microbiological criterion) was used to discriminate between susceptible and resistant organisms. The applied non species-specific veterinary breakpoints are included in Table 2. Herds where no *Pasteurellaceae* were isolated were not included in the further analysis. For each herd an antimicrobial resistance index (ARI) was determined by means of following formula: \(\text{ARI} = y/n \times x\), where \(y\) is the number of resistances detected, \(n\) the number of isolates per herd, and \(x\) the number of antimicrobial drugs tested.\(^{17}\) The Kruskal-Wallis rank test was used to demonstrate differences between herd types based on ARI-values. If significant differences were present (\(p<0.05\)), pairwise comparisons were performed by means of the Mann-Whitney U test. A Bonferroni correction was applied by multiplying \(p\)-values by 3 to adjust for multiple comparisons. All statistical analysis was performed using SPSS, version 11.0 for Windows (SPSS Inc., Chicago, IL, USA).

**RESULTS**

**Bacteriological identification**

*Pasteurella* and/or *Mannheimia* species were isolated from calves in all herds, except for three dairy farms. In total, 222 isolates were obtained from 203 calves (40.9%). Definitive bacteriological results are listed by farm type in Table 1. Final classification by means of 16s rRNA sequence analysis was used for eight isolates initially classified as *Mannheimia haemolytica* sensu lato.

In the dairy herds, a total of 41 *Pasteurellaceae* were retrieved from 35 calves (61.4 %) and further identification resulted mainly in *Pasteurella* (*P.*) *multocida* (\(n=33\)). Other bacteria were, *Mannheimia* (*M.*) *varigena* (4), *M. haemolytica* (sensu stricto) (2), *P. trehalosi* (1), and one not further specifiable *Mannheimia* sp. In six calves, *Pasteurellaceae* isolates of more than one species were found. The seven detected *Mannheimia* isolates originated from five herds. *Mannheimia* spp. were not detected on the two farms where the calves had been vaccinated against *Mannheimia haemolytica*. 

87
On the beef calf farms, a total of 65 Pasteurellaceae were retrieved from 61 animals (40.7%). Molecular identification resulted in 55 P. multocida isolates, although six of them were phenotyped as P. avium biotype 2 (indole negative). For another series of three Pasteurella strains originating from the same herd, further species delineation was not possible by phenotyping or genotyping. The remaining seven Pasteurellaceae, six isolates were haemolytic and designated as M. haemolytica (sensu stricto) and the remaining one as M. varigena (0.7%). In four calves, P. multocida and Mannheimia spp. were isolated together. In two of the three farms where vaccination against Mannheimia haemolytica was performed, Mannheimia haemolytica sensu stricto was isolated.

On the veal calf farms, 116 Pasteurellaceae were detected in 107 calves (37.0%). P. multocida was identified 97 times and thereby the most frequent encountered species. In addition, single isolates of P. aerogenes and P. trehalosi were detected on two farms. Haemolytic Mannheimia organisms were detected in 17 animals (5.9%) from four farms (80.0%). On three of the latter farms, haemolytic Pasteurellaceae were allocated to up to four different species within the genus Mannheimia. Overall, M. haemolytica was isolated eight times, M. glucosida three times, and M. varigena five times. One isolate was highly related (16s rRNA sequence similarities ≥ 99%) to Mannheimia HPA 121 (An'gen et al., 1999). In nine veal calves, isolates of more than one species belonging to the family Pasteurellaceae were isolated simultaneously.

Antimicrobial susceptibility testing

MIC determinations for the reference strains of M. haemolytica sensu stricto, M. glucosida, M. varigena, and Mannheimia biogroup 8A, showed for all compounds the same range (+/− one dilution): ampicillin, 0.25 mg/L; ceftiofur, ≤0.03 mg/L; oxytetracycline, 0.5 mg/L; gentamicin, 1 mg/L; tilmicosin, 8 mg/L; florfenicol, 0.5 mg/L; and enrofloxacin, ≤0.03 mg/L. The same values were found for M. ruminalis and M. granulomatis, with the exception of the MIC of gentamicin for the M. ruminalis type strain (MIC = 0.25 mg/L) and the MIC of tilmicosin (≤ 0.5 mg/L) for the M. granulomatis type strain.
Table 1. Distribution of aerobic *Pasteurellaceae* in the nasopharynx of calves from different bovine herd types.

<table>
<thead>
<tr>
<th>Herd type</th>
<th>Dairy</th>
<th>Beef</th>
<th>Veal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N herds</strong></td>
<td>13</td>
<td>9</td>
<td>5</td>
<td>27</td>
</tr>
<tr>
<td><strong>N calves</strong></td>
<td>57</td>
<td>150</td>
<td>289</td>
<td>496</td>
</tr>
<tr>
<td><strong>N (%) of negative animals</strong></td>
<td>a</td>
<td>22 (38.6)</td>
<td>89 (59.3)</td>
<td>182 (63.0)</td>
</tr>
<tr>
<td><strong>N (%) of calves positive for</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pasteurellaceae</em> (aerobic)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>35 (61.4)</td>
<td>61 (40.7)</td>
<td>107 (37.0)</td>
<td>203 (40.9)</td>
</tr>
<tr>
<td><em>P. aerogenes</em></td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (0.3)</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td><em>Pasteurella</em> spp.</td>
<td>0 (0.0)</td>
<td>3 (2.0)</td>
<td>0 (0.0)</td>
<td>3 (0.6)</td>
</tr>
<tr>
<td><em>P. trehalosi</em></td>
<td>1 (1.8)</td>
<td>0 (0.0)</td>
<td>1 (0.3)</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td><em>Mannheimia (M.) haemolytica</em> sensu lato</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. haemolytica</em> sensu stricto</td>
<td>7 (12.3)</td>
<td>7 (4.7)</td>
<td>17 (5.9)</td>
<td>31 (6.3)</td>
</tr>
<tr>
<td><em>M. glucosida</em></td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>3 (1.0)</td>
<td>3 (0.6)</td>
</tr>
<tr>
<td><em>M. varigens</em></td>
<td>4 (7.0)</td>
<td>1 (0.7)</td>
<td>5 (1.7)</td>
<td>10 (2.0)</td>
</tr>
<tr>
<td><em>Mannheimia</em> spp.</td>
<td>1 (1.8)</td>
<td>0 (0.0)</td>
<td>1 (0.3)</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td><strong>Total N of <em>Pasteurellaceae</em> isolates detected</strong></td>
<td>41</td>
<td>65</td>
<td>116</td>
<td>222</td>
</tr>
</tbody>
</table>

*aBacteriologically negative for *Pasteurellaceae*. bIncluding 6 [*P.* *avium* biotype 2 (indole negative) strains. cSynonym for [*Pasteurella* *haemolytica*].
Of the recovered field strains, a total of 158 Pasteurella and 29 haemolytic Mannheimia isolates underwent susceptibility testing. The remaining Pasteurellaceae strains did not fulfil the CLSI criteria for interpretation of the susceptibility (re-)testing or were lost during storage. The resulting MIC distributions and the applied interpretive criteria are presented in Table 2. For ampicillin, oxytetracycline, and gentamicin, the MICs showed a clear bimodal distribution. Isolates in the higher range of the MICs of the latter three compounds were considered to have acquired resistance. For ampicillin, the MICs of the latter isolates were higher than the CLSI-breakpoints for Haemophilus, whereas for gentamicin, the veterinary-specific CLSI-breakpoints for other bacteria were exceeded. The CLSI breakpoint for ‘organisms other than streptococci’ fitted well for oxytetracycline, but not for the potentiated sulfonamides. For the latter antimicrobial combination, bimodality was only found for Mannheimia organisms and for Pasteurella organisms other than P. multocida. For P. multocida isolates, a less stringent bimodal distribution was found, wherein all strains exhibiting MIC values ≥ 4/76 mg/L for trimethoprim-sulfonamides originated from the veal and beef farms. The only strains showing resistance to enrofloxacin with MIC ≥ 8 mg/L were four Mannheimia isolates from the veal calf herds. In the remaining Mannheimia and Pasteurella isolates, intermediate susceptibility with a MIC range of 0.5-1 mg/L was seen among 17 isolates originating from four veal calf herds. In vitro activity for tilmicosin was limited (MIC ≥32 mg/L) for 20 Pasteurella and 11 Mannheimia strains, all but one originating from veal calf farms. Resistance to florfenicol or ceftiofur was not detected in any of the identified Pasteurellaceae, although one P. multocida isolate from a beef calf showed a decreased susceptibility for ceftiofur (MIC =1 mg/L).

The overall percentages of resistant strains (N strains/total) of the Pasteurellaceae from the dairy, beef, and veal calves were 17.6% (6/34), 21.9% (14/64), and 71.9% (64/89), respectively. In Table 3 the proportion of susceptible isolates and resistance profiles per identified species are listed per herd type. Based on the results of susceptibility testing of 187 Pasteurellaceae, multi-resistance (resistance to more than two antimicrobial compounds) was restricted to the organisms originating from the veal calf farms (Table 3). Only a limited variation in susceptibility profiles among all isolated Pasteurellaceae per herd was detected on the dairy farms (two different profiles on a single farm) and beef farms (two different profiles on six farms, three different profiles on one farm), while a large within-herd variability was found with 3 – 10 different resistance profiles being observed among the five veal herds.
Per herd the antimicrobial resistance index (ARI)-value was calculated based on in vitro activities of eight antimicrobial compounds for all Pasteurella and Mannheimia organisms isolated in that particular farm. The mean ARI-values of the herds belonging to different production types were statistical different (Kruskal-Wallis rank test; p<0.01) (Fig. 1). Further analysis of the mean ARI-values (Mann-Whitney U test) revealed a significant difference between dairy herds and veal calf herds (p<0.01), and between beef herds and veal calf herds (p<0.01).

**Figure 1.** Average antimicrobial resistance index (ARI) per farm type (error bar: range) based on MIC (minimum inhibitory concentration)-determinations of Pasteurella and Mannheimia isolates for 8 antimicrobial drugs. Between brackets: number of herds; number of isolates.
Table 2. Frequency distribution of minimum inhibitory concentrations (MICs) for different antimicrobial agents of 158 *Pasteurella* and 29 *Mannheimia* spp. from calves.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Bacterial species (N isolates)</th>
<th>Number of isolates showing a MIC (mg/L) of&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0.03</th>
<th>0.06</th>
<th>0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>≥128</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ampicillin</strong></td>
<td><em>P. multocida</em> (152)</td>
<td>47</td>
<td>32</td>
<td>51</td>
<td>9</td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pasteurella</em> spp. (6)*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. haemolytica</em> s.s. (15)</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. glucosida</em> (3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. varigena</em> (9)</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Mannheimia</em> spp. (2)</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Ceftiofur</strong></td>
<td><em>P. multocida</em> (152)</td>
<td>81</td>
<td>70</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pasteurella</em> spp. (6)</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. haemolytica</em> s.s. (15)</td>
<td>5</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. glucosida</em> (3)</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. varigena</em> (9)</td>
<td>-</td>
<td>1</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Mannheimia</em> spp. (2)</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Oxytetracycline</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
<td><em>P. multocida</em> (152)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>55</td>
<td>26</td>
<td>7</td>
<td>16</td>
<td>-</td>
<td>2</td>
<td>18</td>
<td>3</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pasteurella</em> spp. (6)</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. haemolytica</em> s.s. (15)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. glucosida</em> (3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. varigena</em> (9)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Mannheimia</em> spp. (2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Gentamicin</strong>&lt;sup&gt;e&lt;/sup&gt;</td>
<td><em>P. multocida</em> (152)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>5</td>
<td>23</td>
<td>50</td>
<td>21</td>
<td>20</td>
<td>3</td>
<td>12</td>
<td>4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pasteurella</em> spp. (6)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. haemolytica</em> s.s. (15)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. glucosida</em> (3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. varigena</em> (9)</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Mannheimia</em> spp. (2)</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>TMP/S</strong>&lt;sup&gt;f,g&lt;/sup&gt;</td>
<td><em>P. multocida</em> (152)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>10</td>
<td>50</td>
<td>18</td>
<td>15</td>
<td>11</td>
<td>7</td>
<td>16</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>Pasteurella</em> spp. (6)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. haemolytica</em> s.s. (15)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. glucosida</em> (3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. varigena</em> (9)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Mannheimia</em> spp. (2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Tilmicosin</strong></td>
<td><em>P. multocida</em> (152)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>6</td>
<td>7</td>
<td>25</td>
<td>25</td>
<td>32</td>
<td>9</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Pasteurella</em> spp. (6)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. haemolytica</em> s.s. (15)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>8</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. glucosida</em> (3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. varigena</em> (9)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mannheimia spp. (2)</td>
<td>P. multocida (152)</td>
<td>Pasteurella spp. (6)</td>
<td>M. haemolytica s.s. (15)</td>
<td>M. glucosida (3)</td>
<td>M. varigena (9)</td>
<td>Mannheimia spp. (2)</td>
<td>P. multocida (152)</td>
<td>Pasteurella spp. (6)</td>
<td>M. haemolytica s.s. (15)</td>
<td>M. glucosida (3)</td>
<td>M. varigena (9)</td>
<td>Mannheimia spp. (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>---------------------</td>
<td>--------------------</td>
<td>----------------------</td>
<td>--------------------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>--------------------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>--------------------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>--------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Florfenicol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Enrofloxacin</strong></td>
<td></td>
<td></td>
<td>60</td>
<td>1</td>
<td>14</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Dotted and full lines indicate applied breakpoints to consider strains intermediate resistant, and resistant, respectively. 

\[25\] **b** CLSI-breakpoints used are for Haemophilus; no Pasteurella- or Mannheimia-specific breakpoints are currently available. 

\[26\] **c** Pasteurella spp. included 1 P. aerogenes, 2 P. trehalosi, and 3 undefined Pasteurella spp. 

\[26\] **d** NCCLS breakpoints used are unspecified veterinary breakpoints. 

\[26\] **e** TMP/S: trimethoprim-sulfonamides (ratio 1/19), only values for trimethoprim are given.
Table 3. Prevalence of acquired resistance to eight antimicrobials among 158 *Pasteurella* and 29 *Mannheimia* isolates recovered from the respiratory tract of calves on dairy, beef, and veal calf herds.

<table>
<thead>
<tr>
<th>Herd type</th>
<th>Bacterial species</th>
<th>Number of isolates with acquired resistance to&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of isolates with absence of resistance/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Am</td>
<td>Otc</td>
</tr>
<tr>
<td>Dairy</td>
<td><em>P. multocida</em></td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td><em>P. trehalosi</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. haemolytica</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. varigena</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Mannheimia spp.</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Beef</td>
<td><em>P. multocida</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Pasteurella spp.</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. haemolytica</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. varigena</em></td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Veal</td>
<td><em>P. multocida</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>P. aerogenes</em></td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>P. trehalosi</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>M. haemolytica</em></td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>M. glucosida</em></td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>M. varigena</em></td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Mannheimia spp.</em></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Am: ampicillin; Otc: oxytetracycline; TS: trimethoprim+sulfonamides; Til: tilimicosin; Ge: gentamicin; Enr: enrofloxacin, no resistance to cefiofur and florfenicol was detected.
**DISCUSSION**

Among the identified *Pasteurella* strains, 5 indole-negative strains were phenotypically identified as *P. avium* biotype 2, but were indistinguishable from *P. multocida* by means of tRNA-intergenic spacer PCR. Since a recent investigation demonstrated that biotype 2 of *P. avium* should be reclassified within the species *P. multocida*, these indole-negative strains were finally categorised as *P. multocida* for further analysis.

In agreement with the present results, earlier investigations on the presence of *Pasteurellaceae* in the nasopharynx of healthy calves documented that *P. multocida* and haemolytic *Mannheimia* organisms ([*P.*] *haemolytica*) can be frequently isolated from the upper respiratory tract of calves. In these older investigations, all haemolytic *Mannheimia* organisms still were identified as [*Pasteurella*] *haemolytica*. Despite that routine laboratories and many peer reviewed studies traditionally still refer to the bulk of haemolytic *Mannheimia* organisms as *M. haemolytica*, referring to the latter group as *M. haemolytica* sensu lato would be more precise. Because the reference strains of the haemolytic *Mannheimia* organisms showed very similar susceptibilities to all compounds tested, this incorrectness is likely to be of minor importance for monitoring antimicrobial resistance. Nevertheless, we demonstrated that besides *M. haemolytica* sensu stricto, other *Mannheimia* organisms were also encountered in the nasal swabs. In addition, all three defined *Mannheimia* species detected in the present study (*M. haemolytica* s.s., *M. varigena*, *M. glucosida*) have already been associated with bovine diseases, suggesting an opportunistic pathogenic behaviour in the respiratory tract, which is typical for many members of the family *Pasteurellaceae*. The large variation within haemolytic *Mannheimia* species was only prominent on the veal calf farms, and is likely to be related to the multi-origin of the animals in this production system.

Since no *Pasteurella*- or *Mannheimia*-specific CLSI-breakpoints are currently available for ampicillin, the potentiated sulfonamides, tetracyclines, and gentamicin, we used the breakpoints for “organisms other than streptococci” in the case of oxytetracycline and potentiated sulfonamides as well as the veterinary-specific breakpoints for *Enterobacteriaceae, Pseudomonas aeruginosa* and *Actinobacillus pleuropneumoniae* in the case of gentamicin. For ampicillin, the CLSI-breakpoints for *Haemophilus* were used as proposed earlier by Schwarz et al.

In the present study the predominant tendency was the substantially higher MICs and
consequently higher frequency of resistance of Pasteurella and Mannheimia organisms in the veal calf herds in contrast with the two other production types. This was seen for ampicillin, tetracyclines, the combination sulfonamides-trimethoprim, tilmicosin, gentamicin and enrofloxacin (Tables 2 & 3). Resistance to these antimicrobial agents has frequently been reported in isolates retrieved from calves suffering from BRD.\textsuperscript{19,23,24,27,30,34} Similar to reports dealing with clinical cases of BRD of the last decade,\textsuperscript{14,27,30} resistance to florfenicol or ceftiofur was not detected in P. multocida and M. haemolytica (sensu lato) strains. However, transferable florfenicol resistance has recently been identified in a bovine Pasteurella multocida isolate from a clinical BRD case.\textsuperscript{18}

Both by MIC determinations and the ARI-values of the Pasteurella and Mannheimia strains, we demonstrated in the present study that the occurrence of multi-resistance within Pasteurellaceae from the upper respiratory tract was restricted to the veal calf farms. In addition to the MIC-determinations, ARI can give additional information on the selection pressure exerted as a whole in a bacterial population because different species can be integrated to calculate an ARI for a certain herd. Although an initial unequal number of calves and farms were included in the study over the different production types (Table 1), the average ARI per herd type was statistically different between the intensively reared veal calves compared to the more extensively loose group housed dairy and beef calves.

In veal calf industry, the routinely administered in-feed medication can exert a selection pressure in the nasopharynx through systemic distribution or through direct contact (nasopharynx or tonsils) with the microbiota of the upper respiratory tract. In both cases, stepwise mutational resistance or horizontal transfer of resistance genes can result in rapid emergence of resistant strains.\textsuperscript{9,29} However, other underlying factors like housing conditions,\textsuperscript{22} age\textsuperscript{9} and milk diet\textsuperscript{21} also might influence the percentage of resistant commensal bacteria in livestock,\textsuperscript{31} irrespectively of the antimicrobial drug use.

In case of a clinical outbreak of BRD, susceptibility profiles of Pasteurellaceae from nose swabs have been proved to reliably guide veterinarians for an appropriate antimicrobial therapy.\textsuperscript{14} The overall low number of resistant strains in dairy and beef herds is in accordance with a former Belgian study in which clinical isolates from animals suffering from BRD were tested.\textsuperscript{10} On the other hand, a multi-resistant P. multocida strain was isolated from an outbreak of pasteurellosis on a Belgian veal calf farm.\textsuperscript{11} These considerations, along with the potential virulence of these bovine Pasteurellaceae,\textsuperscript{19} suggest that the presented data are likely to be
representative for the resistance situation that one can expect in case of clinical outbreaks of pasteurellosis on farms of the respective production types. Clinicians should be aware of the contrast in susceptibility profiles of bovine Pasteurellaceae related to the production type, which was already suggested by an earlier Dutch investigation. Because of the variability both in bacterial species and resistance profiles in such production units, sampling the nasal flora of several affected animals might improve the choice of an efficacious antimicrobial agent in case of therapy failure, relapse or future outbreaks. In respect of this, antimicrobial resistance monitoring programmes that screen bovine Pasteurellaceae might improve their clinical usefulness by providing the production type from which the samples originate.

In summary, the most striking finding from the present study was the conspicuously high multi-resistance rates of P. multocida and haemolytic Mannheimia strains isolated in veal calves, contrary to those isolated from calves housed under less intensive dairy and beef production systems.

**ACKNOWLEDGEMENTS**

This work is supported by the Institute for the Promotion of Innovation by Science & Technology Flanders (Grant no IWT/SB/11134) and the Belgian Federal Public Service of Public Health, Food Chain Safety and Environment (S6144). The authors are very grateful for the excellent assistance of M. Baele, S. Haelterman, A. Van De Kerckhove, and J. Mollet.

**REFERENCES**


26. National Committee for Clinical Laboratory Standards. 2004. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; informational supplement, M31-S1, National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania, USA.


TET(L)-MEDIATED TETRACYCLINE RESISTANCE
IN BOVINE MANNHEIMIA AND PASTEURELLA ISOLATES

Corinna Kehrenberg,\textsuperscript{a} Boudewijn Catry,\textsuperscript{b} Freddy Haesebrouck,\textsuperscript{c}
Aart de Kruif,\textsuperscript{b} Stefan Schwarz\textsuperscript{a}


\textsuperscript{a} Institut für Tierzucht, Bundesforschungsanstalt für Landwirtschaft (FAL),
31535 Neustadt-Mariensee, Germany
\textsuperscript{b} Department of Obstetrics, Reproduction and Herd Health,
\textsuperscript{c} Department of Pathology, Bacteriology and Poultry Diseases,
Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium
Chapter 2.2.3.
ABSTRACT

Objectives: Tetracycline resistant *Mannheimia* and *Pasteurella* isolates, which were negative for the tetracycline resistance genes (*tet*) commonly detected among these bacteria, were investigated for other *tet* genes present and their location.

Methods: *Mannheimia* and *Pasteurella* isolates were investigated for their MICs of tetracycline and their plasmid content. Identification of *tet* genes was achieved by PCR. Plasmids mediating tetracycline resistance were identified by transformation and hybridization experiments. Plasmid pCCK3259 from *Mannheimia haemolytica* was sequenced completely and analysed for its structure and organization.

Results: All tetracycline resistant isolates carried the gene *tet*(L) either on plasmids or on the chromosome. Two *M. haemolytica* isolates and one *Mannheimia glucosida* isolate harboured a common 5.3-kb *tet*(L) plasmid, designated pCCK3259. This plasmid was similar to the *tet*(B)-carrying tetracycline resistance plasmid pHS-Tet from *Haemophilus parasuis* and the aadA14-carrying plasmid pCCK647 from *P. multocida* in the parts coding for mobilization functions. The *tet*(L) gene was closely related to that of the *Geobacillus stearothermophilus* plasmid pTB19. However, the translational attenuator responsible for the tetracycline-inducible expression of *tet*(L) was missing in plasmid pCCK3259. A recombination site was identified downstream of *tet*(L), which might explain the integration of the *tet*(L) gene region into a basic pCCK3259 replicon.

Conclusion: A *tet*(L) gene was shown to be responsible for tetracycline resistance in *Mannheimia* and *Pasteurella* isolates. This report demonstrates an efficient lateral transfer of a tetracycline efflux gene in Gram-negative bovine respiratory tract pathogens, likely originating from Gram-positive bacteria.
INTRODUCTION

The tetracycline resistance gene tet(L) codes for a membrane-associated efflux protein composed of 14 transmembrane segments. It has been detected on plasmids and on the chromosome of members of various Gram-positive genera. Analysis of tet(L)-carrying plasmids from Bacillus, Staphylococcus, Streptococcus, and Enterococcus revealed that these plasmids varied distinctly in their sizes and occasionally also carried additional resistance genes. This observation supported the assumption that interplasmid recombination events may play an important role in the spread of the tet(L) gene. A model for such a recombination event has already been described and supported by sequence data. Expression of tet(L) is usually inducible via translational attenuation. The corresponding regulatory region upstream of the tet(L) gene consists of a reading frame for a small peptide of 20 amino acids and three pairs of inverted repeated sequences which can form different mRNA secondary structures in the absence or presence of tetracyclines, thereby allowing or preventing the translation of the tet(L) transcripts. In contrast to numerous reports on the occurrence of this gene in Gram-positive bacteria, it has rarely been detected in Gram-negative bacteria. So far, its occurrence has been reported in Fusobacterium and Veillonella, and more recently also in Morganella morganii and Actinobacillus pleuropneumoniae. Studies on the expression of a cloned tet(L) gene from Staphylococcus hyicus revealed that this gene was active in Escherichia coli, although the MICs were distinctly lower than in the staphylococcal host and induction was not detectable.

To date, very little is known about the presence and functional activity of tetracycline efflux genes of Gram-positive bacteria in Gram-negative pathogens. In this study, we investigated six naturally occurring, tet(L)-carrying isolates of the bovine respiratory tract pathogens Mannheimia haemolytica, Mannheimia glucosida, and Pasteurella multocida with particular reference to the plasmid location of the tet(L) gene and its genetic environment.
MATERIAL & METHODS

The six Pasteurella and Mannheimia isolates were obtained from nasal swabs of six-weeks old male Holstein Friesian calves from a veal calf farm in Belgium in 2003. The calves were housed within one stable. They had received a prophylactic medication consisting of colistin (polymyxin E), oxytetracycline and flumequin for 13 days. Immediately thereafter, the calves received an oral treatment with sulfonamides and trimethoprim for another four days. The application of antimicrobial agents was stopped approximately three weeks prior to sampling. Species identification was performed as previously described. Resistance phenotypes were determined by disk diffusion and MICs of tetracycline by the broth macrodilution according to the NCCLS document M31-A2 with Staphylococcus aureus ATCC29213 as quality control strain. PCR analyses for the tet genes of hybridisation classes A-E, G, H, K, L, M, and O followed previously described protocols. PCR products were confirmed by restriction analysis. Plasmid preparation by alkaline lysis and electrotransformation experiments into P. multocida P4000 were conducted as described. Transformants were selected on blood agar plates (Oxoid, Wesel, Germany; 5% v/v sheep blood) supplemented with 5-10 mg/L tetracycline. Plasmid DNA obtained from the transformants was subjected to restriction mapping. Sequence analyses were started with four oligonucleotide primers derived from the tet(L) gene sequence from S. hyicus (accession no. X60828). Another six primers designed from the sequences obtained with the aforementioned primers were used to complete the sequencing of the tet(L) plasmid pCCK3259 (MWG, Ebersberg, Germany). Sequence comparisons were performed with the BLAST programs blastn and blastp (http://www.ncbi.nlm.nih.gov/BLAST/; last accessed 23rd April 2005) and with the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html; last accessed 23rd January 2005). The nucleotide sequence of plasmid pCCK3259 has been deposited in the European Molecular Biology Laboratory (EMBL) database under accession number AJ966516. Macrorestriction analysis with Smal and hybridisation studies with a digoxigenin labelled internal 1046-bp BclI fragment of the tet(L) gene from S. hyicus followed previously described protocols.
RESULTS & DISCUSSION

Identification and location of tet genes

The six isolates included in this study originated from different calves of the same farm and included three *M. haemolytica*, one *M. glucosida* and two *P. multocida* isolates. Their MICs of tetracycline ranged between 16 and 64 mg/L (Table 1). PCR screening for the *tet* genes of classes B, G, H and M previously detected among bovine *Pasteurella* and *Mannheimia* isolates revealed no amplicons. Further PCR analysis for *tet* genes of classes A, C, D, E, and O also failed to yield the expected amplicons. However, a PCR assay for the simultaneous detection of *tet(K)* and *tet(L)* revealed the presence of an amplicon of ca. 1.05 kb in all six isolates. Restriction analysis of the amplicon with either *Cla*I or *Bcl*I – restriction sites for both enzymes are located in *tet(L)*, but not in *tet(K)* – resulted in the *tet(L)*-specific fragments of ca. 0.29 and 0.76 kb for *Cla*I and 0.08 and 0.97 kb for *Bcl*I for all isolates tested. One such amplicon from a *M. haemolytica* isolate was sequenced and proved to be 1048 bp in size. It showed a 1-bp difference to the corresponding part of the *tet(L)* sequence of *Geobacillus stearothermophilus* (accession no. M63891). Plasmid location of the *tet(L)* gene was confirmed in two *M. haemolytica* and the single *M. glucosida* isolate by electrotransformation into a *P. multocida* recipient strain and by hybridisation of plasmid profiles with the specific *tet(L)* gene probe. Susceptibility testing of the transformants confirmed that these plasmids conferred only tetracycline resistance (Table 1). The *tet(L)* genes in the remaining *M. haemolytica* and *P. multocida* isolates were assumed to be located on the chromosome. Macrocassette analysis revealed that the *Sma*I patterns of the two *P. multocida* isolates differed by three bands (data not shown). The two *M. haemolytica* isolates that harboured a *tet(L)*-bearing plasmid exhibited the same *Sma*I pattern while the third *M. haemolytica* isolate and the *M. glucosida* isolate showed unique fragment patterns (Table 1).
Table 1. Characteristics of the *Mannheimia* and *Pasteurella* isolates included in this study

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Bacterial species</th>
<th>PFGE patterns</th>
<th>Resistance phenotype*</th>
<th>MIC&lt;sub&gt;Tc&lt;/sub&gt; (mg/L)</th>
<th>Location of the tet(L) gene</th>
<th>Resistance phenotype of transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>2512/2</td>
<td><em>M. haemolytica</em></td>
<td>A</td>
<td>Tc, Amp, Cm, Gm, Km, Sm, Sul, Tmp</td>
<td>32</td>
<td>chrom.</td>
<td>-</td>
</tr>
<tr>
<td>3242/2</td>
<td><em>M. haemolytica</em></td>
<td>B</td>
<td>Tc, Sm, Sul</td>
<td>64</td>
<td>pCCK3259</td>
<td>Tc</td>
</tr>
<tr>
<td>3259/2</td>
<td><em>M. haemolytica</em></td>
<td>B</td>
<td>Tc, Amp, Cm, Sm</td>
<td>64</td>
<td>pCCK3259</td>
<td>Tc</td>
</tr>
<tr>
<td>3250/2</td>
<td><em>M. glucosida</em></td>
<td>C</td>
<td>Tc, Sm, Sul</td>
<td>16</td>
<td>pCCK3259</td>
<td>Tc</td>
</tr>
<tr>
<td>2481/2</td>
<td><em>P. multocida</em></td>
<td>D</td>
<td>Tc, Gm, Km, Sm, Sul</td>
<td>16</td>
<td>chrom.</td>
<td>-</td>
</tr>
<tr>
<td>1007/2</td>
<td><em>P. multocida</em></td>
<td>E</td>
<td>Tc, Gm, Km, Spc, Sm, Sul</td>
<td>32</td>
<td>chrom.</td>
<td>-</td>
</tr>
</tbody>
</table>

*Abbreviations: Amp (ampicillin), Cm (chloramphenicol), Gm (gentamicin), Km (kanamycin), Sm (streptomycin), Spc (spectinomycin), Sul (sulfonamides), Tc (tetracycline), Tmp (trimethoprim).
Structure and organisation of the \textit{tet}(L)-carrying plasmid pCCK3259

The \textit{tet}(L)-carrying plasmids of the two \textit{M. haemolytica} and the \textit{M. glucosida} isolates were subjected to restriction analysis with 19 different endonucleases. Since the three plasmids proved to be indistinguishable by their restriction patterns, a common designation, pCCK3259, was chosen. The plasmid of one of the \textit{M. haemolytica} isolates was sequenced completely and proved to be 5317 bp in size. A search for open reading frames led to the detection of three reading frames for mobilization proteins (Fig. 1a). The \textit{mobC} reading frame coded for a protein of 102 amino acids which showed 95% and 91% identity to the MobC proteins from the \textit{P. multocida} plasmid pCCK647 (accession no. AJ884726) and the \textit{Haemophilus parasuis} plasmid pHS-Tet (accession no. AY862435), respectively. The largest reading frame coded for a 468 amino acid MobA protein which revealed 89% identity to MobA from pHS-Tet and 79% identity to MobA from pCCK647. Within the \textit{mobA} gene, there was a reading frame for a 160 amino acid MobB protein which showed 88% and 86% identity to the MobB proteins from plasmids pHS-Tet and pCCK647, respectively.

Further downstream of \textit{mobA}, the reading frame for a 458 amino acid tetracycline efflux protein of hybridization class L was detected (Fig. 1a). The TetL protein was indistinguishable from most TetL proteins deposited in the databases, including those from \textit{G. stearothermophilus} plasmid pTB19 (accession no. M63891), \textit{Bacillus cereus} plasmid pBC16 (accession no. NP_043524), \textit{Streptococcus agalactiae} plasmid pLS1 (accession no. NP_040422), or \textit{Enterococcus faecalis} plasmid pAM\textalpha{}1 (accession no. NP_863350). TetL from pCCK3259 differed by one amino acid from the TetL proteins of \textit{Enterococcus faecium} (accession no. AY081910) and \textit{G. stearothermophilus} plasmid pTHT15 (accession no. M11036) and by eight amino acid substitutions from that of the \textit{S. hyicus} plasmid pSTE1. The entire \textit{tet}(L) upstream part in pCCK3259 (positions 2973 – 3483) differed distinctly from that of the corresponding \textit{tet}(L) upstream sequences known from Gram-positive bacteria and showed no homology to sequences deposited in the databases. The translational attenuator usually present in the \textit{tet}(L) upstream region was lost completely in the \textit{M. haemolytica} plasmid pCCK3259. However, a putative promoter with a –35 region (TAGACA, positions 3368-3373), a –10 region (TTTAAT, positions 3395-3400) and G at position 3408 as a potential start for transcription of \textit{tet}(L) was detected in this region. Moreover, a suitable ribosome binding site, AGAAGG (positions 3475-3480), was located 6 bp upstream of the \textit{tet}(L) translational start codon GTG. Homology to known \textit{tet}(L)
genes started 3 bp upstream of this start codon. Downstream of the tet(L) gene, a Rho-independent transcriptional terminator consisting of a pair of imperfect inverted repeats of 12 bp and followed by a stretch of seven thymine residues was detected. Homology to known tet(L) downstream sequences ended 193 bp downstream of the tet(L) stop codon at the sequence TTTTATTC (positions 5049-5056). This sequence was identified by sequence comparisons as a potential recombination site (Fig. 1b) which might have played a role in the integration of the tet(L) gene area into a basic pCCK3259 replicon.

The results of this study showed that tet(L) genes are also present in Mannheimia and Pasteurella isolates. Even if they do not confer high-level tetracycline resistance as in the Gram-positive hosts, these genes are expressed in Mannheimia and Pasteurella – despite the lack of the translational attenuator – and allow the bacteria to survive in the presence of the tetracycline levels achievable by application of tetracyclines to the calves. The results of macrorestriction analysis strongly suggested that tet(L)-carrying Pasteurella and Mannheimia strains are spread between the calves within this specific farm. The detection of plasmid pCCK3259 in strains of M. haemolytica and M. glucosida also confirmed horizontal transfer of this plasmid between members of different Mannheimia species.
Figure 1. (a) Schematic presentation of plasmid pCCK3259 from *M. haemolytica* in comparison to plasmids pHS-Tet from *H. parasuis* and pCCK647 from *P. multocida*. The reading frames are shown as arrows with the arrowhead indicating the direction of transcription [rep: plasmid replication; mobA, mobB, and mobC: plasmid mobilization; aadA14: resistance to spectinomycin and streptomycin; tet(B), tet(L): tetracycline resistance]. A distance scale in kb is shown below each map. The grey-shaded areas indicate the areas of ≥90% nucleotide sequence identity between the different plasmids. Restriction sites are abbreviated as follows: B (BclI), Bg (BglII), C (ClaI), D (DraI), E (EcoRI), EV (EcoRV), H (HindIII), Hp (HpaI), K (KpnI), P (PstI), and X (XbaI). Since the sequence of plasmid pHS-Tet has been deposited in the database in a different orientation as compared to plasmids pCCK3259 and pCCK647, the map of pHS-Tet had been re-drawn to better illustrate the areas of homology. Hence the distance scale and the positions of the reading frames in the map of pHS-Tet do not correspond to those in the respective database entry.

(b) Potential recombination site (shown in the box) downstream of tet(L) in pCCK3259 (accession no. AJ966516) and comparison with the corresponding sequences of plasmid pTB19 from *G. stearothermophilus* (accession no. M63891) and plasmid pHS-Tet from *H. parasuis* (accession no. AY862435). The numbers refer to the nucleotide positions in the respective database entries. Vertical bars indicate matching nucleotide sequences.
ACKNOWLEDGEMENTS

We thank Vera Nöding and Roswitha Becker for excellent technical assistance.

REFERENCES


CHAPTER 2.2.4.

FATAL PERITONITIS IN CALVES
CAUSED BY A MULTI-RESISTANT P. MULTOCIDA CAPSULAR TYPE F

Boudewijn Catry, a Koen Chiers, b Stefan Schwarz, c
Corinna Kehrenberg, c Annemie Decostere, b Aart de Kruif a


a Department of Obstetrics, Reproduction and Herd Health,
b Department of Pathology, Bacteriology and Poultry Diseases,
Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium
c Institut für Tierzucht, Bundesforschungsanstalt für Landwirtschaft (FAL),
31535 Neustadt-Mariensee, Germany
Chapter 2.2.4.
ABSTRACT

A fatal case of atypical septicaemia of pasturellosis in veal calves is described. The causative organism was identified as a multi-resistant *Pasteurella multocida* capsular type F isolate. The outbreak was characterized by a fibrinous peritonitis and mortality which are hitherto unreported features of *P. multocida* capsular type F infections.
CASE REPORT

Male Holstein Friesian calves of two weeks of age were housed in individual wooden straw-bedded boxes. They were fed a milk replacer diet twice a day supplemented with 1.5 g oxytetracycline (Oxytem® 80%, Ecuphar) and 0.5 g colistin (polymyxin E) (Promycine Pulvis® 4,800 IE/kg, VMD) for the first five days. At the time of investigation (December 16th, 2003), 180 five to six weeks old calves were present in the herd. The calves had not received any vaccination since the day of arrival at the farm. Three calves had died in the night of December, 15th 2003, one of which was subjected to necropsy on the farm and showed an extended peritonitis. The remaining two calves were submitted within 8 h to the Department of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine at Ghent University for necropsy. About fifteen calves in the boxes next to those of the dead calves showed nasal discharge and a mild diarrhoea. Every calf (ca. 50 kg) of the entire herd was then orally treated (methaphylaxis) with 0.8 g amoxicillin (Dokamox® 80%, Emdoka) twice a day for five consecutive days. Symptoms disappeared within two days without relapses or deaths. Routine laboratory investigation consisted of a direct identification test for antigens of Rota- and Coronavirus, E. coli F5, and Cryptosporidium parvum (Bio-X Digestive ELISA kit; Bio-X Diagnostics) in the faeces of one calf, and detection of Bovine Viral Diarrhoea-antigens by means of real-time PCR (Adiavet®BVD Realtime, Adiagène) in pooled blood samples from 8 calves out of the same herd. All these laboratory tests were negative.

During necropsy of the two calves, samples from cerebrum, cerebellum, brainstem, lung, mesenteric lymph nodes, synoviae of several joints and omentum major were taken and processed according to standard techniques for histological examination. In both calves gross lesions were similar and consisted of an exudative fibrinous peritonitis (Fig. 1). The synoviae of the metacarpal, metatarsal and elbow joint were hyperaemic. The mesenteric lymph nodes were enlarged and mildly haemorrhagic. At histology, the propria of the omentum was oedematous and infiltrated by moderate numbers of neutrophils. The mesothelium was covered with fibrin. The synoviae were hyperemic and oedematous. In one calf, a mild interstitial pneumonia was present. Lesions were not found in all other samples.
Samples of lung, peritoneal fluid and elbow joint were bacteriologically examined using routine standard techniques for aerobic and anaerobic bacteria, but also *Mycoplasma* spp. (16). In both calves, mucoid non-haemolytic Gram-negative bacteria were isolated as abundant pure cultures from peritoneal fluid. In addition, morphologically similar bacteria were abundantly detected in the lung and elbow joint of one calf. Phenotypic bacteriological analysis (1) of one isolate from each sample revealed that these isolates could be assigned to the species *P. multocida* subspecies *gallicida*. The species identification was confirmed by molecular techniques including tRNA-PCR (1), a *P. multocida*-specific PCR and a multiplex PCR for the detection of capsular types (20). The protocol for the capsule multiplex PCR was slightly altered from that described previously (20): bacterial DNA samples were initially denaturated at 95°C for 5 min, followed by 30 cycles of denaturation for 1 min at 95°C, annealing at 55°C for 1 min, extension at 72°C for 1 min, a final extension at 72°C for 7 min, and cooling by 4°C. The tDNA-PCR and the *P. multocida*-specific PCR confirmed the species assignment *P. multocida*, whereas the capsule PCR confirmed the presence of capsular type F in all isolates (Fig. 2).

*Figure 1.* Opened abdomen, left lateral view. Extended adhesive fibrinopurulent peritonitis. 1, left posterior lung; 2, diaphragm; 3, spleen; 4, omentum.
Figure 2. Agarose gel electrophoresis of generated PCR products for *P. multocida* capsular type F. Lane M, molecular size marker (1-kbp DNA ladder, Gibco-BRL-Eggenstein); line 1, negative control; lane 2, positive control; lane 3, isolate from lung (calf 1); lane 4, isolate from elbow joint (calf 1); lane 5, isolate from abdominal fluid (calf 1); lane 6, isolate from abdominal fluid (calf 2).

*In vitro* susceptibility testing was performed by the agar dilution method according to NCCLS document M31-A2 (12, 18) and showed that all *P. multocida* capsular serotype F isolates were susceptible to florfenicol (Minimum Inhibitory Concentration (MIC) 0.25 µg/ml), ampicillin (MIC 0.25 µg/ml) and ceftiofur (MIC ≤0.06 µg/ml); intermediately susceptible to enrofloxacin (MIC 1 µg/ml), and resistant to oxytetracycline (MIC 64 µg/ml), erythromycin (MIC 8 µg/ml), tilmicosin (MIC 32 µg/ml), trimethoprim-sulfamethoxazole (MIC 6.75/128 µg/ml), gentamicin (MIC > 128 µg/ml), and spectinomycin (MIC > 128 µg/ml). Plasmid analysis revealed that the *P. multocida* strain of capsular type F carried a single plasmid of 5.2 kb. Electrotransformation into the plasmid-free and antibiotic susceptible *P. multocida* strain P4000 was conducted as previously described (14) and *in-vitro* susceptibility testing of the corresponding transformants revealed that this plasmid mediated resistance to spectinomycin, but not to sulfonamides, trimethoprim, erythromycin, tilmicosin, tetracycline, nor gentamicin.

**DISCUSSION**

*P. multocida* is an opportunistic pathogen present on mucous membranes of many animal species. The bacterium predominantly causes respiratory diseases and septicaemia
which have been correlated in different animal species with one of the five recognised
capsular serogroups (A, B, D, E, and F) (17). Evidence is present that host predilection and
pathogenesis are linked with certain capsular serogroups (4). Two well documented bovine
syndromes with a high morbidity and mortality are associated with *P. multocida*. *P. multocida*
isolates displaying capsular type A and to a lesser extent D are worldwide
associated with bovine enzootic bronchopneumonia (BEB) or pneumatic pasteurellosis,
whereas isolates of capsular types B and E are well documented in haemorrhagic
septicaemia (HS) in cattle and water buffaloes in tropical regions of predominantly Asia
and Africa (12, 16, 17, 19, 21). BEB is frequently encountered in Belgium and surrounding
countries (2). It is characterized by depression, fever, loss of appetite, nasal discharge and
respiratory symptoms. Mortality is in general low, but concurrent *Mannheimia haemolytica*
infections can result in an increase in mortality (19). Gross findings are mainly a
fibrinopurulent bronchopneumonia and lymphadenitis (22). In contrast, HS is a fatal
septicaemic disease characterized by fever and sudden death (12, 19). Gross lesions consist
of oedema in head region and (less frequently) bleeding from body orifices (19). In the
present case, septicaemia was observed in the calves and lesions were different from those
observed in BEB and HS.

In septicaemic calves, the predominantly isolated bacteria are coliforms,
*Clostridium perfringens* type C, *Salmonella* spp., streptococci, *Mycoplasma* spp. and
*Pasteurellaceae* (3). *Pasteurella* infections in cattle are recognised to be multifactorial with
involvement of viruses (12). However, since *P. multocida* was isolated as a pure culture
and lesions indicative for viral infections were not present at post-mortem examination, it
may be assumed that the *P. multocida* capsular type F was the primary causative agent.
The systemic manifestation might have led to endotoxaemia which then could explain the
acute fatal course, as can be observed in HS (8, 11).

Molecular confirmation is of utmost importance in pathological studies of
*Pasteurellaceae*. Wilson *et al.* (23) demonstrated that conventional serotyping is unreliable
for the identification of *Pasteurella multocida* isolates. Therefore, in the present study the
identification was based on two different molecular assays. *P. multocida* capsular type F
isolates are predominantly retrieved from diseased poultry, in particular turkeys (19), with
a relative frequency among avian strains of 14% in comparison with other capsular types in
Europe (5). Type F isolates occasionally have been reported in ruminants (8, 17), but
information of their origin or pathology involved is still missing. Recent work in the UK by
Davies and colleagues demonstrated that only one out of 153 bovine (6) and two out of 158
porcine (7) *P. multocida* strains could be assigned to capsular type F. While these two porcine isolates were associated with pneumonia, the single bovine strain was isolated from a calf with severe head and periocular oedema, resembling conjunctivitis in poultry. Therefore, and based on the unique genotype of the latter organism and epidemiological considerations (indirect contact with turkeys), an avian origin was attributed to this bovine isolate. In the present report evidence of contact with turkeys was not found, however ostriches were also housed on the farm. Virulent *P. multocida* strains (not capsular typed) have been reported in ostriches (9). Therefore, it can not be ruled out that the bovine *P. multocida* capsular type F strains were of avian origin. This is further suggested by the subspecies of the *P. multocida* strain, namely *gallicida*, which is a typical avian subspecies (16).

Vaccination against *P. multocida* can be achieved using whole-cell bacterins. However efficacy is limited and restricted to the homologous serotype (5, 12, 13, 19). If cases of *P. multocida* capsular type F-associated septicaemia further emerge, the presence of serotype F as a virulent contributor should be taken into account during the development of bovine pasteurellosis vaccines. The close relationship between capsular serotypes A and F (20), possibly related to similar immunogenic structures like outer membrane proteins (5), may result in a certain degree of cross-protection between these serotypes.

A sufficiently high curative dose of antimicrobial drugs is recommended in cases of bovine septicaemia (3). Plasma concentrations are likely to be appropriate in order to obtain inhibitory concentrations of a certain antimicrobial drug in the case of septicaemia. In agreement with the results of *in vitro* susceptibility testing, successful therapy consisted in the present case of in-feed medication with amoxicillin. If no acquired resistance is present, good alternatives may be in-feed administration of tetracycline, the 16-ring macrolide tylosin, or the combination trimethoprim-sulfonamides. Systemic administration of newer molecules like third generation cephalosporins (ceftiofur, cefquinome) or florfenicol, a fluorinated derivative of chloramphenicol, is also indicated (2, 12). The multi-resistant nature of the *P. multocida* isolates is worrisome. According to the farmer, the ostriches housed on the same farm had not received any antimicrobial treatment. It therefore might be a reflection of the high selection pressure exerted in the Belgian veal calf industry by means of starter rations and in-feed medication. The plausible horizontal transfer of a multi-resistance plasmid could, however, not be confirmed by the applied transformation experiments. The location of the remaining resistance genes, except the one encoding for spectinomycin resistance, is therefore likely to be chromosomal.
In conclusion, this is the first report describing a case of septicaemia in calves caused by an uncommon multi-resistant *P. multocida* capsular type F isolate.

**ACKNOWLEDGEMENTS**

This study was supported by the Institute for the Promotion of Innovation by Science & Technology Flanders grant number IWT/SB/13134. J. Mollet and A. Van de Kerckhove are acknowledged for excellent technical assistance.

**REFERENCES**


11. Horadagoda, N. U., J. C. Hodgson, G. M. Moon, T. G. Wijewardana, and P. D. Eckersall. 2001. Role of endotoxin in the pathogenesis of haemorrhagic septicaemia in the b...


CHAPTER 2.2.5.

NOVEL SPECTINOMYCIN/STREPTOMYCIN RESISTANCE GENE, AADA14, FROM P. MULTOCIDA

Corinna Kehrenberg,a Boudewijn Catry,b Freddy Haesebrouck,c Aart de Kruif,b Stefan Schwarz a


a Institut für Tierzucht, Bundesforschungsanstalt für Landwirtschaft (FAL), 31535 Neustadt-Mariensee, Germany
b Department of Obstetrics, Reproduction and Herd Health,
c Department of Pathology, Bacteriology and Poultry Diseases,
Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium
Chapter 2.2.5.
**ABSTRACT**

A novel spectinomycin/streptomycin resistance gene, designated *aadA14*, was detected on the mobilizable 5198-bp plasmid pCCK647 from *Pasteurella multocida*. The *aadA14* gene encodes an aminoglycoside adenylyltransferase of 261 amino acids. Sequence comparisons revealed that the AadA14 protein showed less than 60% identity to the so far known AadA proteins.
INTRODUCTION

Spectinomycin is an aminocyclitol antibiotic which inhibits bacterial protein biosynthesis by reversibly binding to the 30S ribosomal subunit. Resistance to spectinomycin is commonly due to enzymes which inactivate the drug by adenylylation. At least two major groups of adenylyltransferases (AAD) – also known as nucleotidyltransferases (ANT) – involved in spectinomycin resistance can be differentiated. One group consists of enzymes [referred to as AAD(3’’)(9) or ANT(3’’)(9)] which adenylylate spectinomycin at the 9-OH position of the spectinomycin actinamine ring, but also adenylylate the aminoglycoside antibiotic streptomycin at the 3-OH position of the streptomycin glucosamine ring and thereby mediate combined resistance to spectinomycin and streptomycin (39). Such enzymes, of which a considerable number of variants has been described, are known to occur in a wide variety of Gram-negative bacteria, and occasionally also in Gram-positive bacteria such as Enterococcus faecalis (4).

The corresponding genes, which are commonly referred to as aadA or ant(3’’)I, have been detected on plasmids and in the chromosomal DNA with many of them being located on gene cassettes in class 1 integrons (27, 29, 30). A second group of adenylylating enzymes, including those encoded by the genes spc from transposon Tn554 (19) or aad9 from the E. faecalis plasmid pDL55 (14), exhibits only AAD(9) [or ANT(9)] activity and hence confers only resistance to spectinomycin.

In veterinary medicine spectinomycin is commonly used to control bovine respiratory tract infections due to Pasteurella multocida, Mannheimia haemolytica, or Histophilus somni. Although P. multocida and M. haemolytica isolates which exhibit high-level resistance to spectinomycin with MICs of ≥ 256 µg/ml have recently been reported from Germany, attempts to identify aadA, spc or aad9 genes in these isolates failed, as did experimental approaches to horizontally transfer the potential spectinomycin resistance genes (31). In the present study, we identified a first aadA gene on a small plasmid from a bovine P. multocida isolate from Belgium.

DESCRIPTION OF THE AADA14 GENE

The ca. 5.2-kb plasmid pCCK647 was identified in a previously reported P. multocida capsular type F strain which was obtained from a case of fatal peritonitis in calves (3). The plasmid was transferred by electrottransformation into the recipient strains
P. multocida P4000 (18) and E. coli JM109 (Stratagene, Amsterdam, The Netherlands) where it mediated resistance to spectinomycin (MIC ≥ 512 µg/ml) and streptomycin (MIC 256 µg/ml). Since PCR detection for the known spectinomycin/streptomycin or spectinomycin resistance genes (31) yielded negative results, it was assumed that plasmid pCCK647 harboured a so far undescribed type of spectinomycin/streptomycin resistance gene. To identify the resistance gene located on this plasmid, pCCK647 was subjected to restriction mapping (Fig. 1) and ClaI-EcoRI fragments of ca. 0.8 and 4.4 kb were cloned into pBluescript II SK+ (Stratagene, Amsterdam, The Netherlands). Both fragments were sequenced completely on both strands by primer walking starting with the M13 forward and reverse primers (MWG, Ebersberg, Germany).

**Figure 1.** Comparison of the maps of plasmid pCCK647 from P. multocida with the maps of plasmid pHs-Tet from H. parasuis (13) and pAB2 from M. haemolytica (37). The arrows indicate the extents of the genes tet(B) (tetracycline resistance), aadA14 (spectinomycin/streptomycin resistance), rep (plasmid replication), blaROB-1 (ampicillin resistance), mobA, mobB, and mobC (plasmid mobilization) with the arrowheads showing the directions of transcription. The regions of similarity between pCCK647, pHs-Tet and pAB2 are marked by grey shading. A distance scale in kb is given below each map. Restriction endonuclease cleavage sites are abbreviated as follows: Bg (BglII), C (ClaI), D (DraI), E (EcoRI), EV (EcoRV), Hp (HpaI), P (PstI), Pv (PvuII), and X (XbaI).

Sequence analysis identified five open reading frames with one of them exhibiting...
similarity to a plasmid replication gene, three reading frames resembling plasmid mobilization genes, and the remaining one coding for an adenylyltransferase (Fig. 1). The putative rep gene of plasmid pCCK647 coded for a protein of 108 amino acids which showed 57% identity to a 61-amino acid segment of the 94-amino acid replication protein RepB from *Rhodococcus erythropolis* (accession no. AAG29855). A 2680-bp region of pCCK647 comprising the three reading frames for mobilization proteins showed 86.6% and 86.4% similarity to the corresponding regions of the recently described tetracycline resistance plasmid pHS-Tet from *Haemophilus parasuis* (13) and the β-lactamase encoding plasmid pAB2 from *Mannheimia haemolytica* (37), respectively (Fig. 1). The smallest of the three reading frames coding for a 102-amino acid MobC protein overlapped the mobA reading frame by 3 bp. MobC from pCCK647 exhibited 88% identity to the 101-amino acid MobC proteins from plasmids pHS-Tet and pAB2. The 160-amino acid MobB protein showed 89% identity to the 160-amino acid MobB protein from pHS-Tet and 91% identity to the N-terminal 84 amino acids of the 90-amino acid MobB protein from pAB2. The largest reading frame in pCCK647 coded for the 474-amino acid MobA protein. This protein exhibited 79% identity to the 468-amino acid MobA protein from pHS-Tet and 86% identity to the N-terminal 313 amino acids of the 376-amino acid MobA protein from pAB2. Since the mob genes of pCCK647 differed from the ones previously described, mobilization of plasmid pCCK647 was experimentally confirmed. The conjugal tet(A)-carrying tetracycline resistance plasmid pEC1591 originally isolated from *E. coli* and obtained from the strain collection of our institute was chosen to provide the transfer apparatus for the mobilization of plasmid pCCK647. For this, plasmid pCCK647 was first transformed into *E. coli* JM109 which carried the conjugal plasmid pEC1591. Conjugation experiments into the rifampicin resistant *E. coli* strain HK225 (21) by filter mating followed a previously described protocol (8). Transconjugants were selected on triple selective Luria Bertani agar plates supplemented with rifampicin (100 µg/ml), tetracycline (15 µg/ml) and spectinomycin (50 µg/ml). Plasmid analysis and determination of the resistance phenotype of the transconjugants confirmed that the transconjugants carried both plasmids, pEC1591 and pCCK647, and were resistant to rifampicin, tetracycline, streptomycin and spectinomycin. This observation suggests that the mobilization system of plasmid pCCK647 is functionally active.
Figure 2. Homology tree of selected AadA proteins involved in combined resistance to spectinomycin and streptomycin based on a multi-sequence alignment produced with the DNAMAN software (Lynnon-BioSoft, Ontario, Canada). The bacterial source and the database accession number are given for each AadA protein. For a number of AadA proteins, e.g. AadA1 or AadA2, a large number of identical or closely related sequences from different bacterial sources are deposited in the databases. To reduce the complexity of this homology tree, only one representative for each type of AadA protein was chosen. The designations of the different AadA proteins were used as they are deposited in the databases, although these designations do not always reflect the real structural similarities between the different AadA proteins (9). Abbreviations [including reference to the corresponding AadA proteins if published] are as follows: A. baumannii, Acinetobacter baumannii [AadA – 20, AadA4 - 32]; A. genomosp. 3, Acinetobacter genospecies 3 [AadA1 – 40]; A. hydrophila, Aeromonas hydrophila; C. jejuni, Campylobacter jejuni [AadA2 – 22]; C. freundii, Citrobacter freundii [AadA2 – 24]; C. glutamicum, Corynebacterium glutamicum [AadA2 – 34, AadA9 – 33]; E. faecalis, Enterococcus faecalis [AadA – 4]; E. coli, Escherichia coli [AadA1 – 16, AadA2 – 28, AadA4 – 1, AadA5 – 36]; K. oxytoca, Klebsiella oxytoca [AadA – 26]; K. pneumoniae, Klebsiella pneumoniae [AadA8 – 25]; P. multocida, Pasteurella multocida [AadA1 – 38]; P. aeruginosa, Pseudomonas aeruginosa [AadA1 – 15, AadA6 – 2, AadA10 – 23]; S. Agona, Salmonella enterica serovar Agona [AadA23 – 17]; S. Choleraesuis, Salmonella enterica serovar Choleraesuis; S. Newport, Salmonella enterica serovar Newport [AadA7 – 6]; S. Typhimurium, Salmonella enterica serovar Typhimurium [AadA1 – 35, AadA21 – 7]; S. flexneri, Shigella flexneri; uncult. bacterium, uncultured bacterium, V. cholerae, Vibrio cholerae [AadA2 – 5].
The fifth reading frame in pCCK647 coded for a (3")(9) adenylyltransferase of 261 amino acids, designated AadA14. Comparisons with other AadA proteins on the basis of a multisequence alignment revealed an overall low degree of 51.4% to 56.5% to the currently known AadA proteins with the best matches to the AadA23 protein from *Salmonella enterica* serovar Agona (17) and its close relative Aad23b from *E. coli* (accession no. BAD38865). The corresponding homology tree shown in Fig. 2 confirms that AadA14 is only distantly related to the other AadA proteins and clusters with them at 57% identity. In the sequences flanking the *aadA14* gene, neither relics of integron sequences, nor sequences resembling a 59-base element or parts of the 3' conserved segments of class 1 or class 2 integrons (27) were detectable. Thus, it is unlikely that the *aadA14* gene is a cassette-borne *aadA* gene.

To determine whether the gene *aadA14* also occurs in other epidemiologically unrelated high-level spectinomycin/streptomycin resistant *Pasteurella* and *Mannheimia* isolates, an *aadA14*-specific PCR assay was developed. The primers *aadA14*-fw 5'-TCACTTGTGTGGTCCGCAGT-3' and *aadA14*-rev 5'-TCTTTCCGGATAAGCTGCCAGA-3' (annealing temperature 60 °C) were used to amplify an internal 642-bp fragment of the *aadA14* gene. Moreover, this amplicon was cloned into pCR-Blunt II Topo (Invitrogen, Groningen, The Netherlands), cut off from the vector by EcoRI digestion, labelled with the Dig-High Prime DNA Labelling and Detection Starter Kit I (Boehringer, Mannheim, Germany) and used as a gene probe for Southern blot hybridization of HindIII-digested whole cell DNA (10, 11). Three *P. multocida* and two *M. haemolytica* isolates from Germany (31), all exhibiting MICs of spectinomycin of ≥ 256 µg/ml and MICs of streptomycin of ≥ 128 µg/ml, were investigated for the presence of the gene *aadA14*. Another 11 bovine *P. multocida* isolates which exhibited only spectinomycin resistance, seven from Germany and four from Belgium, were also included. However, negative results were obtained with both methods for all 16 isolates tested. The PCR-based observation that the four Belgian isolates also did not carry so far known *aadA* genes or the genes *spc* and *aad9* is in agreement with previously published findings on the German isolates (31). Attempts to detect the spectinomycin adenylyltransferase gene *aadA* from *Legionella longbeachae* (accession no. AF288536) and the aminocyclitol/aminoglycoside phosphotransferase gene *aph*(9)-Ia from *Legionella pneumophila* (accession no. U94857) also yielded negative results for all 16 isolates. These results strongly suggest that so far undescribed genes are responsible for spectinomycin and spectinomycin/streptomycin resistance in *Pasteurella* and *Mannheimia* organisms. Moreover, the results of this study
and another recently published study (12) show that Pasteurella isolates carry certain resistance genes that are distantly related to genes from other bacteria which mediate the same resistance phenotype.

**Nucleotide sequence accession number.** The sequence of the 5198-bp plasmid pCCK641 has been deposited in the EMBL database under accession number AJ884726.

**ACKNOWLEDGEMENTS**

The authors thank Vera Nöding and Roswitha Becker for excellent technical assistance.

**REFERENCES**


CHAPTER 3.

General Discussion
Chapter 3.
GENERAL DISCUSSION

To treat bovine pasteurellosis and in a larger context bovine respiratory disease, the currently most effective method is the use of antimicrobial drugs (Kehrenberg et al., 2001b). Over time, the use of antimicrobial drugs inevitably leads to the selection of resistant bacteria. Because of the public health hazard involved (through zoonotic pathogens or horizontal transfer of resistance genes), several international agencies encourage the policy of a prudent use of antimicrobial drugs to minimize resistance selection (Mevius et al., 1999; Avorn et al., 2001). In general terms, a prudent use firstly implies that one should keep the etiologic agent and its intrinsically or possible acquired resistance profile in mind. Secondly, a prudent use must be interpreted as minimizing the selection pressure predominantly by reducing the use of antimicrobial agents. Translated to bovine pasteurellosis, one needs to document the resistance profiles of the Pasteurellaceae involved, and one should consider under which circumstances antimicrobials are mostly applied, respectively. Only then, concrete precautionary guidelines can be proposed and implemented. Because resistance profiles are variable over time and geography (Catry et al., 2003), temporal and local data are warranted.

Resistance reports of bovine pathogenic Pasteurellaceae have since long been published on a national or regional basis. However, the family Pasteurellaceae underwent several taxonomical reclassifications during the last decade. Our findings provide evidence that older reports on acquired resistance in organisms formerly known as [Pasteurella] haemolytica, still are valuable for comparative purposes, because the distinction between haemolytic Mannheimia species is not a prerequisite for a reliable susceptibility report. For detailed epidemiological or molecular purposes, delineation of Pasteurellaceae up to (sub)species level is however advisable. One such differentiation method here applied is tRNA-intergenic spacer PCR (tDNA-PCR). This technique proved to be a rapid and valuable tool for species delineation, with the exception of M. haemolytica, M. glucosida, and M. ruminalis (Chapter 2.1). Although a first distinction can be made based on the presence or absence of haemolysis on sheep blood agar, one should remind that haemolysis can be lost during subculturing (Barbour et al., 1997). Because both M. haemolytica and M. glucosida are frequently isolated from bovine pneumatic lungs (Angen et al., 2002), the clinical relevance of this distinction needs further attention in respect of pathogenesis studies and vaccine development.
Whereas currently no other PCR techniques have been applied for the identification of Mannheimia organisms, many have been used for the delineation of Pasteurella organisms. Most attention has been paid to the identification of P. multocida (Kasten et al., 1997; Townsend et al., 1998, Miflin and Blackall, 2001; Petersen et al., 2001). In the latter studies, organisms formerly known as Bisgaard taxon 13 (P. canis biovar 2 and [P.] avium biovar 2) were identified as P. multocida. This result was identical to our tDNA-PCR results of several [P.] avium biovar 2 strains recovered from beef calf herds, which were indistinguishable from P. multocida fingerprints (Chapter 2.2.2). Recently, Bisgaard taxon 13 has been included within the species P. multocida (Christensen et al., 2004). These findings suggest that the discriminatory power of tDNA-PCR is comparable to aforementioned molecular tools. In addition, the most outstanding advantage is the possibility to screen large sets of unrelated organisms belonging to different genera, and the interlaboratory exchangeability of tDNA-fingerprints (Baele et al., 2001). Nevertheless, tDNA-PCR failed to give a clear identification for a minority of haemolytic Mannheimia isolates. In these cases, 16S rRNA sequence analysis was used. Hence, evidence was provided for the presence of so far unassigned taxa belonging to the genus Mannheimia in Belgian bovine herds. These results are in line with a previous Danish (Angen et al., 2002) and Australian report (Blackall et al., 2001).

For taxonomical studies, which are still ongoing to further clarify the complex family of Pasteurellaceae, the standard molecular methods (DNA-DNA hybridisations and 16S rRNA sequence analysis) are currently also combined with specific PCRs or sequence analysis of house-keeping genes and virulence genes. Currently, this approach has been used for the genus Actinobacillus (Christensen and Bisgaard, 2004), although miscellaneous classified organisms were still reported. In one particular study (Chapter 2.2.4.), we used the multiplex-PCR developed by Townsend et al. (2001) for confirmation of the identification and for capsular typing of a P. multocida isolate. By means of this multiplex-PCR the presence of species specific and capsular type specific molecules can be investigated simultaneously. However, accuracy can be improved by performing the species specific PCR and capular type multiplex-PCR serially instead of simultaneously (Schwarz S., personal communication, 2005), which unfortunately makes the methodology more expensive and time consuming for screening large sets of bacteria.

The predominant capsular type of P. multocida associated with diseases in calves in Western Europe is type A, and to a lesser extent type D. During our studies, a P. multocida
capsular type F strain was recovered from several animals suffering from peritonitis and sepsis on a veal calf herd (Chapter 2.2.4). It would be interesting to capsular type the *P. multocida* isolates collected during the different studies described in this thesis. This would allow obtaining better insights in the prevalence of type F strains in healthy and diseased calves. Studies comparing virulence of type F, A and D strains are also of interest and might allow to identify specific virulence factors in the septicaemic strains.

Remarkably, in human medicine the number of reports on *Pasteurella* infections is increasing over recent years, and even without a history of animal exposure it is thought that an animal reservoir is the major source (Fajfar-Whetstone *et al.*, 1995; Ashley *et al.*, 2004). Cases of severe peritonitis associated with *P. multocida* are infrequently, but regularly reported in humans, especially in immunocompromised persons (suffering from liver cirrhosis) with a documented mortality exceeding 30% (Raffi *et al.*, 1987; Ashley *et al.*, 2004, Tamaskar and Ravakhah, 2004). Unfortunately, none of these isolates has been capsular typed, impairing further comparisons with veterinary medicine.

In spite of recent advantages in molecular techniques, first screening and presumptive identification of *Pasteurellaceae* will remain to rely on morphology, restricted phenotyping and knowledge of the animal species involved (Christensen and Bisgaard, 2004). Haemolysis and indole production are key phenotypical characteristics to distinguish *Pasteurella* (non-haemolytic, indole positive) from *Mannheimia* (haemolytic, indole negative) (Angen *et al.*, 1999). Yet, these two characteristics can be misleading for final delineation. Examples of this are *M. ruminalis* which is not haemolytic and the indole-negative *P. multocida* variants formerly classified as Bisgaard taxon 13.

To facilitate the detection and identification of *Pasteurellaceae* during the here presented studies, a selective medium containing bacitracin was positively evaluated for the isolation of *Pasteurellaceae* from nasal swabs. A similar medium is used in human medicine for the isolation of *Haemophilus influenzae* (Nye *et al.*, 2001). Other successfully used selective media for the isolation of *P. multocida* contain besides bacitracin also gentamicin and other antimicrobial agents (Moore *et al.*, 1994; Muhairwa *et al.*, 2001). Because of the possibility that even low concentrations of gentamicin can interfere with the growth and thereby the detection of susceptible *Pasteurella* and *Mannheimia* organisms, these media were not used. Based on preliminary unpublished results, disinfection of the nostrils should be encouraged when examining the nasal flora of calves. Contamination of the samples is then minimized, which can also be obtained by the use of guarded swabs. In
the larger context of antimicrobial resistance, it is noteworthy that in human medicine, nasal swabbing currently became the standard procedure to investigate the carrier state of patients in respect of nosocomial infections (Troche et al., 2005).

From an epidemiological and surveillance point of view, antimicrobial resistance has been widely investigated in both zoonotic bacteria and commensal intestinal microbiota from livestock. Less attention has been paid to the commensal respiratory microbiota (Sørum and Sunde, 2001). In the intestinal microbiota, predominantly enterococci and *E. coli* have been studied and compared among different animal species. Enterococci and *E. coli* represent indicator bacteria in a certain ecological niche, hereby reflecting the selection pressure on Gram-positive and Gram-negative bacteria, respectively. The three criteria, on which the choice of these indicator bacteria was made, are their abundant presence in healthy target animals, their easy standard bacteriological culture conditions, and their acceptability for resistance genes (Catry et al., 2003). As expected from previous reports, the predominant organism found in the upper respiratory tract of calves was *P. multocida*. One could evaluate the use of *P. multocida* as indicator bacterium, and firstly conclude that this organism meets the three criteria mentioned above (Kehrenberg et al., 2001b; Ashley et al., 2004). An additional relevant valuable characteristic is its potential pathogenicity in almost all livestock production animal species (cattle, poultry, swine, rabbits) (Kehrenberg et al., 2001b) and in humans (Ashley et al., 2004). Finally, *P. multocida* is an interesting indicator bacterium because of its natural antimicrobial susceptibility profile. Although it is a Gram-negative bacterium, it is naturally susceptible for antimicrobial classes like penicillins and macrolides, which, generally speaking, cover a Gram-positive (and anaerobic) spectrum.

An ever returning point of discussion when monitoring programmes are critically reviewed is the sample strategy (Davison et al., 2000). More exactly, how many isolates, animals, and farms should be sampled to get an accurate estimate of the resistance situation in a certain geographical area? For the intestinal indicator and zoonotic bacteria, currently one organism isolated during the slaughter process is examined to represent the resistance situation in a certain herd (Bywater et al., 2004). When focusing on the respiratory tract, a similar procedure would not cover the within-herd variability as found for the *Pasteurellaceae* in the veal calf farms. In the *P. multocida* and *Mannheimia* isolates from the particular veal herd where the tet(L) gene was identified, this variability of both species and resistance profiles was confirmed by means of pulsed-field gel electrophoresis (PFGE).
Interestingly, analogous within-herd variability was recently documented in swine for the zoonotic bacterium *Salmonella Typhimurium* (Nollet *et al.*, 2005). Monitoring programmes should therefore carefully document the applied sampling strategy and discuss possible bias in their reports. Nevertheless, the large variability in resistance percentages of monitored bacteria over time and place (Catry *et al.*, 2003), once again confirms the necessity of regional monitoring programmes.

Antimicrobial resistance monitoring programmes from France (Martel *et al.*, 1995), Germany (Wallmann *et al.*, 2004), and the Netherlands (MARAN-2003) already include *P. multocida* and *M. haemolytica* sensu lato isolates from cattle suffering from respiratory disorders. Veterinarians can consult these reports for choosing an empirical appropriate antimicrobial therapy. In the Belgian pilot monitoring programme supported by the *Belgian Antibiotic Policy Coordination Committee* (BAPCOC), these organisms are planned to be incorporated in the future (BAPCOC, 2003). The results of monitoring programmes should however be interpreted with caution because of the possibility of misidentification and the use of sometimes insufficiently standardized susceptibility testing by means of disk diffusion methods. When performing dilution tests of *Pasteurellaceae* according to the international guidelines of the *Clinical and Laboratory Standard Institute* (CLSI, until January 2005 known as NCCLS), one needs to remind that veterinary breakpoints still require fine-tuning, and that testing is furthermore complicated by the fact that currently *Pasteurella and Mannheimia* should be tested on two distinct media (NCCLS, 2004). For the latter, the influence of a possible misclassification due to misleading absence of haemolysis or indole production needs to be taken into account.

Bovine *Pasteurella and Mannheimia* isolates of monitoring programmes are frequently recovered from fatal respiratory disorders. Since an intensive selection pressure may have been exerted during the course of the therapy, the resistance situation of *Pasteurella and Mannheimia* organisms reported in these programmes might be an overestimation compared to the field situation (Catry *et al.*, 2002; MARAN-2003). In order to clarify and seize such a discrepancy between necropsy and field strains, one should consider the presence of minority populations of resistant strains that become the predominant population during administration of antimicrobial agents. These minority resistant populations might remain undetected during standard bacteriological procedures examining only one purified strain from a particular sample. However, this hypothesis was not supported during the present investigations using tetracycline as a model molecule.
Possible reasons that could have masked the support of this theory are the choice of the molecule and/or target bacteria, the fact that no tonsillar examinations (Kehrenberg et al., 2001b) were included, or the production type investigated (loose grouped calves).

In the studies described in Chapter 2.2.2, a marked difference in occurrence of resistant Pasteurellaceae was found between loose group and individually housed calves at high density. As encouraged in these studies, the Dutch MARAN programme recently made the distinction between isolates recovered from veal calves, and from less intensively housed dairy calves (MARAN-2003). In agreement with our findings, both resistance and multi-resistance were found more frequently in isolates from the veal calves. Since veal calves all originate from less intensively housed cattle farms, the high prevalence of antimicrobial resistance genes is remarkable. Further research is needed to elucidate this particular epidemiology. In this context, one also needs to consider the maintenance of certain clones between consecutive groups of calves within a certain barn or stable. In industrialized raised poultry such persistence has been demonstrated for vancomycin-resistant enterococci, in spite of intensive hygienic measures in between consecutive flocks (Heuer et al., 2002). In less intensively reared livestock, an opposite phenomenon called dilution effect might take place. This consists of the dispersion of resistant strains in a large reservoir of susceptible microbiota. Such susceptible reservoir is present in environmental niches such as pasture, soil and water. A dilution effect in relation to antimicrobial resistance was already suggested by Langlois et al. (1988) who found a higher level of resistant coliforms in finishing unit pigs compared to pigs in the farrowing house or on pasture.

Although several antimicrobial resistance genes of Pasteurella and Mannheimia organisms already have been described, including their localization in the bacterial genome (Kehrenberg et al., 2005), many other resistance genes need to be discovered and explored. This information is essential to identify the origin and the localisation within the bacterial genome required for the emergence and the possible persistence of resistance genes within these opportunistic respiratory pathogens. In swine, there are indications that tetracycline resistant [P.] aerogenes isolates acquired tet(B) genes through horizontal gene transfer from Enterobacteriaceae (Kehrenberg and Schwarz, 2001). In a further study (Chapter 2.2.3) on some of the here reported tetracycline resistant Pasteurella and Mannheimia strains from the veal calves, the resistance gene tet(L) was identified and it was shown to be localised on either a plasmid or the chromosome. Since the tet(L) gene is commonly
found in Gram-positive bacteria (Schwarz et al., 1996), horizontal gene transfer may have taken place into the Gram-negative Pasteurella and Mannheimia bacteria. Such lateral gene exchange has also been documented between enterococci and Enterobacteriaceae (Tenover, 2001). Further dissemination of the tet(L) gene among Pasteurellaceae may have been favoured through plasmid mediated exchange between Pasteurella and Mannheimia species, as already demonstrated for tet(H) (Kehrenberg et al., 2001a). To further reveal the extent of such intergenus flow of resistance genes, investigating simultaneously the Gram-negative and Gram-positive microbiota in a certain ecological niche should be encouraged. For this purpose, promising tools are microarrays that are able to simultaneously detect the presence of up to 90 different resistance genes in organisms belonging to different bacterial species (Perreten et al., 2005).

The tet(B) and tet(L) genes found in Pasteurellaceae thus likely originate from other Gram-negative and Gram-positive bacteria, respectively. Similar to other resistance genes found in Pasteurella and Mannheimia, e.g. sul2 (sulfonamide resistance) and strA (streptomycin resistance), original selection of these resistance genes might have taken place in other bacterial genera. In contrast, resistance genes like tet(H), blaROB (beta-lactamase), floR (florfenicol resistance) (Kehrenberg and Schwarz, 2005b), dfra20 (trimethoprim resistance) (Kehrenberg and Schwarz, 2005a), and the here discovered aadA14 gene (spectinomycin/streptomycin) have not been described in other bacterial families. Thus, the latter series of resistance genes seem to have been more specifically selected in Pasteurellaceae.

At the onset of the presented studies, consumption data of antimicrobial agents in cattle were restricted to one report on Scandinavian dairy herds (Grave et al., 1999). In a survey undertaken at our department during 2002-2004, we quantitatively and qualitatively documented antimicrobial drug consumption on 5 beef, 5 dairy, and 5 veal calf herds (Feyen et al., 2004; 2005). It was concluded that overall average daily treatment incidences per 1000 animals on veal calf farms (range 45.1-369.7) were largely exceeding the treatment incidences found on dairy (3.2-10.9) and beef herds (3.0-10.2), mainly due to oral group treatments (medicated starter rations) in the veal calves during the first weeks of the production cycle. This routine practice was only performed in the veal calf farms, for which the main indication is the prevention of digestive and respiratory disorders. Almost all oral treatments performed in the veal herds (88.0%) were given at a lower daily dosage than recommended in the leaflets of the respective compounds (underdosed), whereas the
parenterally administered antimicrobial drugs were mainly overdosed in all herd types (Feyen et al., 2004; Feyen et al., 2005). Interestingly, the same 5 beef and 5 veal herds were included in our studies (Chapter 2.2.2) for the antimicrobial resistance index (ARI) calculations of the Pasteurellaceae. When these ARI-values are rudimentary compared with the antimicrobial treatment incidences, the highest treatment incidences and lowest dosages were found in the herd type associated with the highest ARI, and vice versa. A weakness of this procedure is that ARI-calculations are highly variable depending on the choice and the number of antimicrobial agents included. In depth analysis is necessary to reveal the extent of the correlation between antimicrobial use and the occurrence of antimicrobial resistance in Pasteurellaceae from the bovine respiratory tract. Nevertheless, the suggested preliminary association is in line with what has been reported for the intestinal microbiota of swine by Dunlop et al. (1998). They found a significantly higher prevalence of resistant faecal coliforms in pigs receiving antimicrobials orally in comparison with individually and systemically treated animals.

As for all infectious diseases, veterinary clinicians are encouraged to take samples for an etiological diagnosis of bovine respiratory disease. For bovine pasteurellosis in particular, reliable samples for susceptibility profiling are bronchoalveolar lavages and nasal swabs taken from affected animals at the onset of an outbreak (DeRosa et al., 2000; Catry et al., 2002). Further research is warranted to confirm that such investigations performed on nasal swabs taken prior to an outbreak in healthy animals in a particular herd, can guide practitioners for an appropriate therapy during future outbreaks of bovine pasteurellosis. Our findings suggest that for this purpose, sampling several animals might enhance the detection of resistant strains because of a possible large within-herd variability of resistance patterns in a certain herd. This is especially the case in veal calf herds.

Grimshaw et al. (1987) experimentally documented that one can expect an increase of approximately 10% in mortality due to bovine pasteurellosis if the causative agent is resistant to the antimicrobial administered. The identification of a potential respiratory pathogen susceptible for a certain antimicrobial, is however no guarantee for clinical cure by administering this antimicrobial agent. Severity of infection, pharmacodynamical and host related factors, or other unidentified pathogens (e.g. Mycoplasma dispar), are possible explanations for therapy failure under such conditions.
Since resistance and multi-resistance were predominantly seen on the veal calf farms, a reduction of the selection pressure exerted by oral group treatments seems appropriate from a public health point of view. However, and as for all intensive livestock production systems, one should thoroughly consider possible economical and animal welfare counterparts of such actions. In the European Union, the number of veal calves slaughtered in 2002 was estimated to be 5.3 million (Desmet S., personal communication, 2004). On the other hand, veal meat is reported to be frequently contaminated with antimicrobial residues (Jemmi and König, 1999), another unfavourable public health aspect of this production type. Our findings therefore support the worldwide call for alternative, non-antimicrobial intervention strategies such as improved vaccination programmes and reduced housing density for the control of infectious diseases in livestock (Avorn et al., 2001).

In summary, five main conclusions can be drawn from the present studies. First, exact speciation may not influence clinical management, but will help to characterize the prevalence, the origin and spread of resistance genes, and the pathogenic potential of Pasteurella and Mannheimia organisms. Second, an etiologic diagnosis of the multifactorial bovine respiratory disease complex can be complicated by a large within-herd variability of potential respiratory pathogens and their resistance profiles. Third, the largest and most heterogenic reservoir of resistant Pasteurella and Mannheimia organisms that might lead to therapy failure during outbreaks of bovine pasteurellosis can be found in the veal calf industry. Fourth and although this needs to be confirmed, the data suggest that a selection pressure exerted by oral group treatments in high density livestock is very efficient in altering the respiratory commensal microbiota, including gene transfer across genus and species borders. Finally, antimicrobial resistance monitoring programmes dealing with Pasteurellaceae should include production type of origin from both a clinical and epidemiological point of view.
REFERENCES


Christensen H., Angen O., Olsen J.E., Bisgaard M. 2004. Revised description and classification of atypical isolates of *Pasteurella multocida* from bovine lungs based on genotypic characterization to include variants previously classified as biovar 2 of *Pasteurella canis* and *Pasteurella avium*. Microbiol. 150: 1757-1767.


Chapter 3.


NCCLS 2004. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; informational supplement, M31-S1, National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania, USA.


Summary
SUMMARY

One of the economically most important diseases in European bovine livestock is pasteurellosis. Bovine pasteurellosis is an element of the multifactorial enzootic bronchopneumonia syndrome that mainly affects calves. Typical symptoms are fever, dyspnoea, cough, nasal discharge and anorexia. The bacteria involved belong to the family Pasteurellaceae and are part of the normal upper respiratory tract. Hence, the disease is difficult to prevent. The current available vaccines are not always effective due to the variety of pathogens and environmental factors involved. For a veterinary practitioner, knowledge of the pathogen involved and its antimicrobial resistance profile is essential information for an accurate treatment of bovine pasteurellosis. An etiological diagnosis can be obtained by means of tracheal washings or, less reliably, with nasal cultures. During the last two decades, several reports have documented antimicrobial resistant strains and resistance genes within bovine Pasteurellaceae isolated from clinical cases. However, a substantial reclassification of this group of bacteria was carried out at the end of the previous century, with the designation of organisms formerly known as [Pasteurella] haemolytica into the new genus Mannheimia. Herein, Mannheimia (M.) haemolytica is the type species besides four other new species. The accuracy of resistance data prior to this reclassification is thus questionable, since intrinsically antimicrobial susceptibilities may vary among different bacterial species belonging to a certain genus. In addition, the reason for a large variation in resistance prevalence of Pasteurellaceae from clinical cases of bovine pasteurellosis over time and geography is not understood. Several antimicrobial resistance genes and their localization in the bacterial genome have been documented, but many remain to be explored. This is essential information to identify the origin and the selection pressure required for the emergence and possible persistence of these opportunistic respiratory pathogens. Once this information is available, clinicians can minimise the number of therapy failures related to antimicrobial resistance in bovine Pasteurellaceae (Chapter 1.1).

The general aim of the present thesis (Chapter 1.2) was to study the occurrence of antimicrobial resistance in potential bovine pathogens belonging to the genera Pasteurella and Mannheimia. Special attention has been paid to the recent reclassifications within this bacterial family, and to tetracycline resistance. Worldwide, tetracyclines are still among the most commonly applied antimicrobial agents, and resistance is frequently encountered in Pasteurellaceae from bovine origin.
Summary

The thesis is further divided into two sections. A first section focuses on the molecular differentiation of Pasteurella and Mannheimia organisms associated with bovine pasteurellosis. In a second section, the occurrence of antimicrobial resistance is investigated in Pasteurellaceae isolated from the nasopharynx of healthy calves and from one atypical clinical case, including the identification and the mechanism of spread of some underlying resistance genes.

For the differentiation of Pasteurella and Mannheimia organisms up to (sub)species level, tRNA-intergenic spacer PCR (tDNA-PCR) was evaluated by means of reference strains and an additional set of clinical field isolates of bovine origin (Chapter 2.1). This PCR fingerprinting method is based upon the amplification of spacers between the transfer RNA genes, using consensus primers complementary to the highly conserved edges of the tRNA genes. tDNA-PCR enabled discrimination of all Pasteurella species (Pasteurella (P.) aerogenes, P. avium, P. canis, P. lymphangitidis, P. multocida, P. trehalosi) tested. For the differentiation of the subspecies of P. multocida, an additional dulcitol reaction was required. Two of the five so far defined Mannheimia species, M. granulomatis and M. varigena, had a distinct fingerprinting profile. The phylogenetically highly related species M. haemolytica, M. glucosida, and M. ruminalis clustered together. Nevertheless, M. ruminalis is non-haemolytic, and M. haemolytica and M. glucosida can be differentiated on the basis of two additional phenotypic characteristics (β-glucosidase and aesculin hydrolysis). It was concluded that tDNA-PCR, expanded with a minimum of additional biochemical tests, enables rapid discrimination of Pasteurella and Mannheimia (sub)species of human and veterinary importance.

The five following studies focus on antimicrobial resistance in Pasteurella and Mannheimia organisms. The first of these surveys (Chapter 2.2.1) was undertaken because isolates from fatal cases (necropsy) of bovine pasteurellosis harbour more often resistance determinants as compared to isolates retrieved from acute cases prior to therapy. We were interested to know whether this discrepancy is due to the presence of undetected resistant subpopulations already present prior to therapy. Following therapy, these subpopulations might either be selected and/or pass their resistance genes through horizontal gene transfer into the predominant pathogenic population. To further elucidate this, the presence of
subpopulations of tetracycline resistant *Pasteurellaceae* in the upper respiratory tract of clinically healthy calves was investigated. Simultaneously, the occurrence of *Pasteurella* and *Mannheimia* organisms in the nasopharynx according to the recent classifications was included as a study objective. For both purposes, nasal swabs were taken from 61 loose group housed calves from 16 herds. All animals were 1 to 4 months old and had no recent history of antimicrobial treatment. Inoculation was performed aerobically on a selective medium (Columbia sheep blood agar with 16 mg/L bacitracin) with or without 4 mg/L oxytetracycline (OTC). *Pasteurellaceae* were isolated from 62.3 % of the calves, and the most predominant were *P. multocida* subsp. *multocida* (57.4%), *M. varigena* (4.9%), and *M. haemolytica* (3.2%). Growth of *Pasteurellaceae* on the OTC-containing medium was only seen with samples from two herds (6 animals; 9.8%), and in only one farm this proved to be an OTC-resistant subpopulation. Minimum inhibitory concentration (MIC) determinations by means of agar dilution confirmed a low prevalence of OTC-resistant *Pasteurellaceae*, with overall MIC$_{50}$ and MIC$_{90}$ values of 0.25 and 32 mg/L, respectively. The results did not support the hypothesis that the relatively high frequency of tetracycline resistant *Pasteurellaceae* from fatal cases of bovine respiratory disease is related to the presence of minor tetracycline resistant subpopulations.

In the next survey (Chapter 2.2.2) we were interested if large variations of antimicrobial resistance in bovine *Pasteurella* and *Mannheimia* organisms could be linked with the production type of origin. Therefore, the presence and antimicrobial susceptibilities (MIC determination by macrodilution for eight antimicrobial agents) of potential pathogenic *Pasteurellaceae* isolated from the nasopharynx of 496 clinically healthy calves on 27 Belgian farms were investigated. The farms covered three production types, namely dairy farms, beef farms, and veal calf farms. To detect intrinsical differences in antimicrobial susceptibilities, all reference strains within the genus *Mannheimia* were included during the susceptibility testing. For all tested compounds, all haemolytic *Mannheimia* reference strains (*M. haemolytica* sensu lato) showed the same MIC-range. It was therefore concluded that exact speciation of haemolytic *Mannheimia* strains may not influence clinical management, although it might be important for epidemiological and pathogenesis studies.

In the nasopharynx of the examined calves, *P. multocida* (37.3%) and *M. haemolytica* sensu lato (6.3%) were the most frequently detected organisms and were overall very susceptible to all antimicrobial agents tested when retrieved from dairy calves.
and beef calves. In contrast, decreased susceptibility for ampicillin, oxytetracycline, potentiated sulfonamides, gentamicin, tilmicosin, enrofloxacin, and multi-resistance was abundantly present among *P. multocida* and *M. haemolytica* s.l. retrieved from the veal calf herds. All isolates tested were susceptible to florfenicol and ceftiofur. The discrepancy between antimicrobial susceptibilities of *Pasteurellaceae* from intensively reared veal, and more extensively reared dairy and beef calves, was statistically confirmed at herd level by means of the antimicrobial resistance index (ARI). The ARI was calculated by means of following formula: $ARI = \frac{y}{n} \times x$, where $y$ is the number of resistances detected, $n$ the number of isolates per herd, and $x$ the number of antimicrobial drugs tested. Finally, a substantial within-herd variability of species diversity and resistance profiles among isolates belonging to the genera *Pasteurella* and *Mannheimia* was found on the veal calf farms. This was attributed to the multi-origin of the animals and the routinely administered in-feed medication typical for this production system. Thus, an etiologic diagnosis of the multi-factorial bovine respiratory disease by means of nose swabs could be complicated by a large within-herd variability of potential respiratory pathogens and their resistance profiles.

A series of six tetracycline resistant *Pasteurellaceae* originating from one of the latter veal calf farms was further examined (Chapter 2.2.3) for the causative underlying resistance genes and their localization in the bacterial genome. The six isolates included three *M. haemolytica*, one *M. glucosida* and two *P. multocida* isolates, and all harboured the gene *tet*(L) either on a plasmid or on the chromosome. This tetracycline resistance gene codes for a membrane-associated efflux protein and is commonly found in Gram-positive bacteria. One 5.3-kb plasmid (pCCK3259) containing the *tet*(L) gene was present in two *M. haemolytica* and one *M. glucosida* strains. The plasmid was sequenced completely and analysed for its structure and organization. Herein, only the parts coding for mobilization functions were similar to those previously found in *Pasteurellaceae*. The translational attenuator responsible for inducible expression of *tet*(L) was absent. These results demonstrated an efficient lateral transfer of a tetracycline efflux gene in Gram-negative bovine respiratory tract pathogens, likely originating from Gram-positive bacteria. Even if they do not confer high-level tetracycline resistance as in the Gram-positive hosts, these genes are expressed in *Mannheimia* and *Pasteurella* – despite the lack of the translational attenuator – and allow the bacteria to survive in the presence of the tetracycline levels achievable by application of tetracyclines to the calves. The results of macrorestriction analysis strongly suggested that *tet*(L)-carrying *Pasteurella* and *Mannheimia* strains spread
between the calves within this specific farm. The detection of plasmid pCCK3259 in strains of *M. haemolytica* and *M. glucosida* also confirmed horizontal transfer of this plasmid between members of different *Mannheimia* species.

In the fourth report, a fatal case of atypical septicaemia of pasteurellosis in veal calves is described (Chapter 2.2.4). The causative organism was identified as a multiresistant *P. multocida* subspecies *gallicida* capsular type F isolate. The outbreak was characterized by fibrinous peritonitis and mortality, which are hitherto unreported features of *P. multocida* capsular type F infections. Additional examinations (Chapter 2.2.5) showed that the causative strain harboured a novel resistance gene, designated *aadA14*, which mediates resistance to spectinomycin and streptomycin. The *aadA14* gene encodes an aminoglycoside adenyltransferase, and sequence comparisons revealed that the AadA14 protein showed less than 60 % identity to the so far known AadA proteins. The *aadA14* gene of the bovine *P. multocida* strain was located on a mobilizable 5198-bp plasmid (pCCK647) which was demonstrated to be functionally active.

Because of the routine practice of in feed medication specifically in the veal calf industry, the overall results suggest that the consequent selection pressure exerted by these oral group treatments in high density livestock is efficient in altering potential pathogenic respiratory commensal microbiota, including horizontal resistance gene transfer even across species or genus barriers. It was further concluded that antimicrobial susceptibility reports of bovine *Pasteurella* and *Mannheimia* organisms should include the production type of origin (Chapter 3). Then, consulting veterinarians can choose a more appropriate empirical therapy for the treatment of bovine pasteurellosis.
Samenvatting
SAMENVATTING

In de Europese rundveehouderij wordt boviene pasteurellose als een economisch belangrijke ademhalingsaandoening beschouwd. Deze ziekte maakt deel uit van het multifactoriële enzootische bronchopneumoniesyndroom dat voornamelijk kalveren treft. De symptomen van dit syndroom zijn koorts, hoest, ademnood, neusuitvloeiing, en een verminderde eetlust. De betrokken bacteriën behoren tot de familie van de Pasteurellaceae en zijn facultatief pathogeen omdat ze aanwezig kunnen zijn in de bovenste ademhalingswegen van gezonde dieren. Preventie van boviene pasteurellose is niet eenvoudig. Omdat ook andere micro-organismen en verschillende omgevingsfactoren betrokken zijn in de pathogenese, geven vaccins niet altijd voldoende bescherming tegen boviene pasteurellose. In geval van een klinische uitbraak, leidt een antimicrobiële therapie tot de beste behandelingsresultaten. Een succesvolle behandeling steunt op de identificatie van de oorzakelijke kiem en haar gevoeligheidspatroon voor de beschikbare antimicrobiële middelen. Een etiologische diagnose kan worden verkregen door middel van longspoelingen of, zij het iets minder betrouwbaar, door middel van nasale swabs. Gedurende de laatste decennia werd vaak resistentie gerapporteerd voor Pasteurellaceae geïsoleerd uit klinische gevallen van boviene pasteurellose. Een recente en drastische taxonomische reclassificatie van deze groep bacteriën, met als voornaamste wijziging de introductie van Mannheimia (M.) haemolytica, stelt de accurate van oudere rapporten in vraag. Elk bacteriële species wordt immers gekenmerkt door een specifieke intrinsieke gevoeligheid ten opzichte van antimicrobiële middelen. Bovendien kent de prevalentie van resistente Pasteurellaceae geïsoleerd uit klinische gevallen een grote verscheidenheid naargelang plaats en tijd. De onderliggende oorzaken van deze variatie zijn onvoldoende bekend. Ook zijn lang niet alle onderliggende resistentiemechanismen en hun localisatie in het bacterieel genoom beschreven. Dit is nochtans essentiële informatie om het ontstaan, de verspreiding en de persistentie van antibioticumresistente Pasteurellaceae te begrijpen. Dergelijke informatie kan de practicus helpen bij het kiezen van een juiste therapie bij pneumonieën die veroorzaakt worden door resistente Pasteurellaceae (Hoofdstuk 1.1.).

De algemene doelstelling van deze thesis (Hoofdstuk 1.2.) was om het voorkomen van antibioticumresistente facultatief pathogene Pasteurellaceae van kalveren te beschrijven, met aandacht voor de recente taxonomische herklassering. Tevens werd speciale aandacht besteed aan tetracyclineresistentie, vermits tetracyclines wereldwijd één van de meest gebruikte antibiotica zijn en resistentie hiervoor vaak gerapporteerd wordt bij
Samenvatting

boviene Pasteurellaceae.

De experimentele studies van deze thesis worden beschreven in twee delen. In het eerste deel wordt de nadruk gelegd op de moleculaire identificatie van Pasteurella en Mannheimia stammen die worden geassocieerd met boviene pasteurellose. Een tweede luik behandelt het voorkomen van antibioticumresistentie bij Pasteurellaceae geïsoleerd uit de bovenste ademhalingswegen van gezonde kalveren en uit een atypische klinische vorm van pasteurellose. Er werd tevens aandacht geschonken aan de identificatie en de verspreidingsmechanismen van enkele onderliggende resistentiegenen.

Door middel van referentiestammen en een reeks bijkomende isolaten uit klinische gevallen van boviene pasteurellose, werd de moleculaire techniek tRNA-intergenic spacer PCR (tDNA-PCR) geëvalueerd als identificatiemiddel voor Pasteurella en Mannheimia species (Hoofdstuk 2.1.). Deze fingerprinting methode berust op de vermenigvuldiging door middel van PCR en detectie van specifieke genetische regio’s tussen de genen die coderen voor transfer-RNA (tDNA). Hiertoe worden universele primers gebruikt die binden op specifieke maar evolutief stabiele uiteinden van het tDNA. Door middel van tDNA-PCR was het mogelijk om alle geteste Pasteurella species (Pasteurella (P.) aerogenes, P. avium, P. canis, P. lymphangitidis, P. multocida, P. trehalosi) duidelijk te onderscheiden. Voor het aantonen van de subspecies van P. multocida, was een bijkomende fenotypische test noodzakelijk (dulcitol suikerfermentatie). Van de vijf tot dusver benoemde Mannheimia species, vertoonden M. granulomatis en M. varigena een duidelijk herkenbaar patroon. De fylogenetisch sterk verwante species M. haemolytica, M. glucosida, en M. ruminalis vormden één grote cluster van atypische patronen. Om deze drie kiemen van elkaar te onderscheiden, kan men zich voorerst baseren op de aan- of afwezigheid van haemolyse (afwezig bij M. ruminalis). Het onderscheid tussen M. haemolytica en M. glucosida kan gemaakt worden aan de hand van het resultaat van twee bijkomende biochemische testen (β-glucosidase en hydrolyse van esculine). Samenvattend, vormde tDNA-PCR in combinatie met een beperkt aantal biochemische testen, een snelle en betrouwbare identificatiemethode voor Pasteurella en Mannheimia species.

De vijf volgende studies hebben betrekking op het voorkomen van antibioticumresistentie bij boviene Pasteurella en Mannheimia stammen. De eerste hiervan (Hoofdstuk 2.2.1.) werd opgezet vanuit de vaststelling dat isolaten van fatale, niet op een
therapie reagerende gevallen van pasteurellose, vaker resistentie vertonen in vergelijking met isolaten van acute gevallen vóór enige behandeling. Een mogelijke hypothese hiervoor was de aanwezigheid van onopgemerkte resistentie subpopulaties die reeds aanwezig zijn voorafgaand aan de behandeling. Tijdens een antimicrobiële therapie kunnen deze subpopulaties uitgeselecteerd worden tot - en/of hun resistentiegenen doorgeven (via horizontale genenoverdracht) naar - de dominante pathogene populatie. Om deze theorie te staven, werd de aanwezigheid van tetracyclineresistente subpopulaties van Pasteurellaceae in de bovenste luchtwegen van klinisch gezonde kalveren onderzocht. Terzelfdertijd werd hierbij het voorkomen van Pasteurella en Mannheimia organismen in de neusholte onderzocht met aandacht voor de taxonomische reclassificaties. Hiertoe werden nasale swabs genomen van 61 kalveren uit loopboxen van 16 rundveebedrijven. Alle dieren waren 1 tot 4 maand oud en waren recent (1 maand) niet behandeld met antibiotica. De stalen werden aëroob geïncubeerd op een selectief medium (bloedagar met 16mg/L bacitracine) met en zonder toevoeging van 4 mg/L oxytetracycline. Uit 62.3% van de kalveren werden Pasteurellaceae geïsoleerd, en de meest voorkomende kiemen waren P. multocida subsp. multocida (57.4%), M. varigena (4.9%), en M. haemolytica (3.2%). Bij 6 dieren (9.8%) van 2 bedrijven werden Pasteurellaceae geïsoleerd op het selectieve medium waaraan oxytetracycline was toegevoegd, en slechts op 1 bedrijf vormden deze kiemen een tetracyclineresistente subpopulatie. Aan de hand van minimum inhibitorische concentratie (MIC) bepalingen met behulp van de agardiluitemethode werd de lage prevalentie van tetracyclineresistente Pasteurellaceae bevestigd. De globale MIC₅₀ en MIC₉₀ waarden bedroegen respectievelijk 0.25 en 32 mg/L. De hypothese dat de relatief hoge frequentie aan tetracyclineresistente Pasteurellaceae van fatale gevallen te wijten zou zijn aan de aanwezigheid van tetracycline resistente subpopulaties, kon niet bevestigd worden.

In een volgend onderzoek (Hoofdstuk 2.2.2.) werd nagegaan of de grote variaties in antibioticumresistentie van bovine Pasteurella en Mannheimia isolaten konden worden gerelateerd aan een bepaald productietype. Hiervoor werden de aanwezigheid en de antibioticumgevoeligheden van potentieel pathogene Pasteurellaceae in de neusholte van 496 klinisch gezonde dieren van 27 bedrijven onderzocht. Drie bedrijfstopes werden hierbij betrokken, met name melkvee-, zoogkoeien-, en dichtbevolkte (intensieve) vleeskalverenbedrijven. De gevoeligheidsbepalingen (MIC bepalingen) gebeurden aan de hand van de macrodilutie-methode voor 8 antimicrobiële producten. Hierbij werden referentiestammen behorende tot het genus Mannheimia opgenomen, teneinde intrinsieke
Samenvatting

differentiate in susceptibility. All haemolytic Mannheimia strains (M. haemolytica sensu lato) showed a similar spread in susceptibility for the tested molecules. Therefore, it was concluded that a precise identification up to species level is not required for the clinical approach of bovine pasteurellosis. Such an identification of haemolytic Mannheimia strains is, however, recommended for epidemiological purposes and for further understanding of the pathogenesis.

In the nostrils of the investigated calves, P. multocida (37.3%) and M. haemolytica sensu lato (6.3%) were most frequently detected. The Pasteurellaceae isolated from the calves of the dairy and mixed farms were generally very susceptible to the tested antimicrobials, in contrast to the strains from the meat calves, where a reduced susceptibility to ampicillin, oxytetracycline, the combination trimethoprim-sulfamid, gentamicine, tilmicosine, enrofloxacine, a multi-resistant, was common. All tested strains were susceptible to ceftiofur and florfenicol. The contrast between the antibiotic susceptibilities of the Pasteurellaceae from one side of the more extensively kept calves, and the other side of the more intensively kept meat calves, was found statistically significant at the farm level by using an antimicrobial resistance index (ARI). For each farm, an ARI was calculated using the following formula: ARI = y/n * x. Here, y is the number of detected resistance determinants, n the number of isolates per farm, and x the number of tested antimicrobial substances. In addition, a remarkable within-farm variability of bacterial species belonging to the genera Pasteurella and Mannheimia, and of the measured resistance profiles was found. Two causes that likely contribute to this variability are the differences in the origin of these calves, and the routine (oral) administration of antibiotics in the meat calf breeding. Consequently, it was suggested that an etiological diagnosis of bovine pasteurellosis by means of nasal swabs can be complicated by this large within-farm variability of potential respiratory pathogens.

In a series of 6 tetracycline-resistant Pasteurellaceae, isolated from a meat calf breeding during the previous experiment, the underlying resistance genes and their localization within the bacterial genome (Chapter 2.2.3) were subsequently searched for. For the relevant M. haemolytica (3), M. glucosida (1), and P. multocida (2) strains, the tet(L) resistance gene was detected both on a plasmid and in the chromosome. This resistance gene codes for a membrane-bound efflux protein, and is often found in Gram-positive bacteria. The tet(L) gene was moreover
ontdekt op een plasmide met een omvang van 5.3 kb (pCCK3259) dat in 2 *M. haemolytica* en 1 *M. glucosida* stam werd gevonden. Het betreffende plasmide werd gesequeneerd. In vergelijking met wat voorheen werd aangetoond in Pasteurellaceae, werden slechts enkele genen verantwoordelijk voor mobilisatie gevonden. De genetische informatie verantwoordelijk voor de inductie van de tetracyclineresistentie (translational attenuator), karakteristiek voor tet(L) genen in Gram-positieve kiemen, werd niet gedetecteerd. Deze resultaten toonden aan dat het tetracycline efflux gen zich efficiënt kan verspreiden tussen bovienne Gram-negatieve ademhalingspathogenen, en dat het vermoedelijk afkomstig is van Gram-positieve bacteriën. De betrokken stammen vertoonden niet de hoge MIC waarden zoals gebruikelijk voor de tet(L)-positieve Gram-positieve gastbacteriën. De tet(L) genen worden evenwel tot expressie gebracht in Mannheimia en Pasteurella, ondanks de afwezigheid van de genetische informatie die codeert voor inductie. Hierdoor worden deze bacteriën niet in groei geremd door tetracyclineconcentraties die normaliter in het lichaam van een kalf kunnen worden bereikt. Typering van de stammen met behulp van pulse field gel electrophoresis (PFGE), toonde aan dat Pasteurella en Mannheimia stammen die het tet(L) gen bevatten, zich efficiënt verspreiden tussen verschillende kalveren van hetzelfde bedrijf. De aanwezigheid van het plasmide pCCK3259 in zowel *M. haemolytica* als *M. glucosida*, doet ten sterkste vermoeden dat er een plasmidaire overdracht tussen verschillende Mannheimia species had plaatsgevonden.

In het op één na laatste onderzoek (Hoofdstuk 2.2.4.) wordt een uitbraak van een atypische septicaemische vorm van bovienne pasteurellose bij vleeskalveren beschreven. De oorzaakelijke kiem werd geïdentificeerd als *P. multocida* subspecies *gallicida* kapseltype F, en was multi-resistent. De ziekte werd gekenmerkt door een fibrineuze peritonitis en sterfte, wat tot dusver nog niet was beschreven bij infecties veroorzaakt door kapseltype F stammen van *P. multocida*. Verder onderzoek (Hoofdstuk 2.2.5.) toonde aan dat de betrokken *P. multocida* stam een nog niet eerder beschreven resistentiegen bevatte dat gelocaliseerd was op een plasmide. Dit plasmide (pCCK647) was mobiliseerbaar, functioneel actief, en had een omvang van 5198 baseparen. Het zich hierin bevindende structurele gen, *aadA14* genoemd, codeerde voor een aminoglycoside adenyltransferase en resulteerde in resistentie voor zowel spectinomycine als streptomycine. Het betreffende AadA 14 proteïne vertoonde minder dan 60% gelijkenis met de tot dusver bekende AadA proteïnen.
Samenvatting

Vanwege het routinematig toedienen van antibiotica aan vleeskalveren via het voeder, en op grond van de globaal verkregen gegevens (Hoofdstuk 3.), werd geconcludeerd dat de antibioticumresistentie-selectiedruk in de intensieve vleeskalverhouderij op een efficiënte wijze de potentieel pathogene microbiota in de bovenste luchtwegen van kalveren kan beïnvloeden, inclusief een interspecies en intergenerische overdracht van resistentiegenen. Tenslotte moet op basis van deze resultaten worden aangeraden om, tijdens het rapporteren van gevoeligheden van boviene Pasteurella en Mannheimia stammen, het productietype van oorsprong te vermelden. Hierdoor zal de praktizerende dierenarts een gerichtere empirische therapie kunnen kiezen.
Curriculum vitae
CURRICULUM VITAE

Boudewijn Catry was born on July 23rd 1976 in Diest, Belgium, and finished high school in 1994 at the Montfortcollege of Rotselaar, a pleasant boarding school near to Leuven. Memories of his childhood centred on his brother and parents, football, music, and youth movements. He graduated in 2000 from the Faculty of Veterinary Medicine at the University of Ghent, after several memorable and valuable student activities.

At the Faculty of Veterinary Medicine, he has been working from 2000 onwards as a PhD-student at the department of Reproduction, Obstetrics and Herd Health. In addition, he also participates in student’s education in the ambulatory clinic of the Veterinary school, paying extra attention to antimicrobial therapy, respiratory disorders, and obstetrics. In 2001, he received a grant from the Institute for the Promotion of Innovation by Science and Technology Flanders. His main area of research is antimicrobial resistance in livestock, with emphasis on bovine respiratory pathogens, and he is an invited member of the veterinary division of the Belgian Antibiotic Policy Coordination Committee. A close cooperation with the department of Pathology, Bacteriology and Poultry diseases of the Faculty of Veterinary Medicine and with the German ‘Institut für Tierzucht, Bundesforschungsanstalt für Landwirtschaft (FAL)’ of Neustadt-Mariensee, allowed him to finish this doctoral thesis dealing with bovine Pasteurellaceae.

Since 2003, he has been married to Hilde Demarré. She sometimes likes to listen attentively while he plays music. Hilde also gave birth to his brown-eyed daughter Hannelore. So far, both Hilde & Hannelore make him smile over and over again…
BIBLIOGRAPHY

Articles


Publications in proceedings of national and international congresses


Curriculum vitae


Concluding Remarks
CONCLUDING REMARKS

All of this work is collaborative, therefore the author wants to thank all co-authors, colleagues, veterinarians, students, technicians, and farmers, which were indispensable for the present thesis and other studies included in the bibliography. The author also likes to acknowledge the important contributions made to the drafting of this thesis by Stefan Schwarz, Dick Mevius, Piet Deprez, Patrick De Backer, Annemie Decostere, Luc Devriese, Siska Croubels, Lieven De Zutter, Geert Opsomer, and Marc Coryn. This list is far from complete without my excellent supervisors Aart de Kruif and Freddy Haesebrouck.

The financial and logistic support from the University of Ghent, the Institute for the Promotion of Innovation by Science & Technology Flanders, the Belgian Federal Public Service of Public Health, Food Chain Safety, and Environment, the Flanders Animal Health Service, in particular Mia Vanrobaeys, and the Belgian Antibiotic Policy Coordination Committee are also much appreciated. The author is also deeply grateful for the support and generosity of Stefan Schwarz and Corinna Kehrenberg from the German Institut für Tierzucht, Bundesforschungsanstalt für Landwirtschaft (FAL) of Neustadt-Mariensee.

The author further acknowledges with gratitude the colleagues from the department of Reproduction, Obstetrics, and Herd Health, from the department of Pathology, Bacteriology and Poultry Diseases, from the department of Internal Medicine and Clinical Biology of Large Animals, and from the department of Pharmacology, Pharmacy and Toxicology for the pleasant collegial atmosphere and company during congresses. The author also wishes all Belgian bovine practitioners pleasant evolutions with regard to the profession.

Finally, my deepest sympathy goes out to my excellent supervisors, and to Stefan Schwarz, Hans Laevens, Jan Govaere, Tom Vanholder, Steven Verberckmoes & Leen Vandaele, Bart Mateusen, Tom Meyns, Jeroen Dewulf, Geert Hoflack, Jozef Laureyns, Dries Everaert, Caroline Bauwens, Annemie Decostere, Koen Pattyn, Arlette van de Kerckhove, Serge Verbanck, Sofie Haelterman, Els Defré, Roger Verbeke, Vera Nöding & Roswitha Becker, my friends from Kortrijk, Rotselaar & Betekom, many of their relatives, my family in law, my parents, my brother Frederick and his family, and especially to my wife Hilde Demarré and our daughter Hannelore. It is an honour to share memories with all of you.

Sincerely yours,

Boudewijn
Concluding Remarks

CODE:

The quick brown fox jumps over the lazy dog